PRECONCEPTION CHRONIC MALE ALCOHOL EXPOSURE INDUCES FAS LIKE GROWTH RESTRICTION

A Thesis

by

SARAH KATHERINE CHRONISTER

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Michael Golding
Committee Members,	Shannon Washburn
	Rajesh Miranda
Head of Department,	Larry Suva

August 2016

Major Subject: Biomedical Sciences

ABSTRACT

Epidemiologic studies demonstrate that alcohol is the most prevalent teratogen to which humans are exposed. In the US, it is estimated that at least one percent of children suffer from fetal alcohol spectrum disorder (FASD) or alcohol-related birth defects associated with Fetal Alcohol Syndrome (FAS). FAS is associated with facial abnormalities, cognitive impairment, behavioral problems, and decreased birth weight. Over the past 40 years, clinical studies have reported multiple instances where infants were born with characteristic FAS symptoms to mothers who had not consumed alcohol during pregnancy; but whose fathers were either heavy drinkers or chronic alcoholics. These studies indicate that the father's lifestyle may be an unrecognized element in the genesis of this disorder. Studies of a wide-range of environmental exposures have demonstrated that changes in the male-inherited epigenetic program profoundly influence offspring development and health. Given that 70% of men drink and 40% drink heavily, we hypothesize that errors in the male-inherited developmental program drive several key FAS-associated birth defects.

We examined how chronic preconception male alcohol exposure impacts fetal growth and development. We employed a mouse model that allowed us to quantitatively track parental patterns of inheritance. B6(Cast7) males were chronically exposed 10% ethanol for a period of 70 days, and then mated with non-exposed C57BL/6J dams. Our studies identified a significant decrease in the weight of the gestational sac and fetus, as

well as a decrease in crown rump length. While placental weights were identical between the treatment groups, we did identify a significant increase in the relative placental weight of offspring sired by alcohol exposed males. In addition, we found that many of the growth defects exhibited a female sex-specific patterns of inheritance with three parameters that were measured. Molecular analysis of of multiple candidate imprinted genes identified alteration in the expression of H19, Cdkn1c, Dio3 and Mirg. Of these, Dio3 displayed a sex-specific effect, where treated females exhibited increased expression compared to control females as well as male offspring from treated males. Collectively, our studies suggest that chronic preconception male alcohol exposure is associated with fetal growth restriction, a well-characterized FAS-associated birth defect.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Epigenetics

1.1.1 The History of Epigenetics

Epigenetics is the study of heritable changes in gene expression and cellular phenotypes that occur through modifications to the genome caused by environmental factors, but which do not change the underlying DNA sequence. The term epigenetics stems from the Greek word "epi", meaning above, therefore, epigenetics is said to be above genetics. This field of epigenetics originated by observing inheritance patterns which contradicted Mendelian genetics, such as variation in embryo growth, twins, and plants [1] (Figure 1).

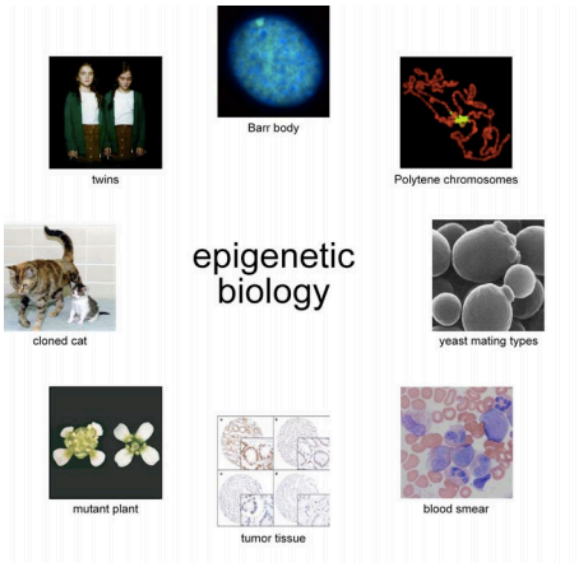


Figure 1. Examples of Epigenetic Phenotypes. Twins, silenced X chromosome, polytene chromosome, yeast mating types, blood smear, tumor tissue, mutant plant, and cloned cat. [1]

The study of epigenetics seeks to understand how zygotes develop into an organism with specific phenotypes. There are several questions scientists are studying. Among these are: How could the phenotype change without the change of underlying nucleic acids [2]? What controls genes being expressed while others are silent? How does the environment play a role in phenotypic expression?

The first studies of epigenetics were performed by H.J. Muller using X-rays and the Drosophila model. He found a class of alleles that had a high rate of phenotypic change which he called “eversporting displacements”. The most prominent phenotypic change was in the eyes, where he noticed their eyes had a mosaic patterning: “even if all the parts of the chromatin appeared to be represented in the right dosage - though abnormally arranged” [3].

Additionally, in 1951 Barbara McClintock, discovered that “controlling elements” were the cause of the different physical characteristics in maize [4]. McClintock identified two genetic loci that were dominant in determining the kernel colors in maize, Dissociator (Ds) and Activator (Ac). She discovered that these loci could transpose on the chromosome resulting in a mosaic color pattern (Figure 2). In the presence of Ac, the Ds can break away from chromosome 9. When Ds breaks away, the aleurone-color gene is released and the synthesis of pigment can begin resulting in the mosaic color pattern.

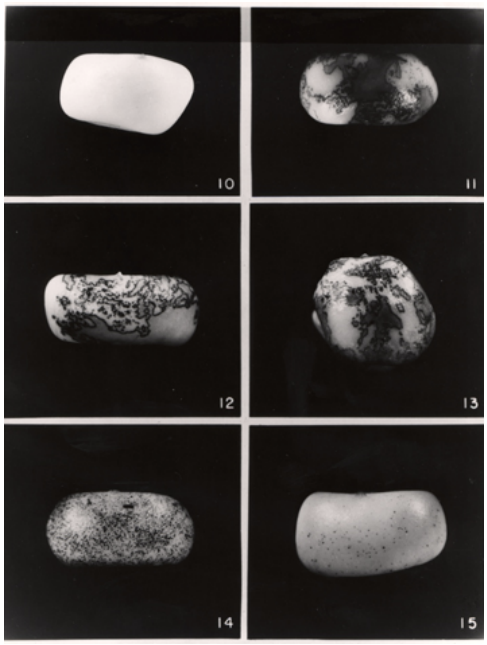


Figure 2. Mosaic Coloring of a Kernel. There is no Ac is present in 10 and Ds inhibits the synthesis of color. (11-13) One Ac is present and Ds moves to create a mosaic pattern by synthesizing pigments. (14) Two Ac are present (15) Three Ac are present. [4]

Another study examined the inactivated X chromosome and found an epigenetic process [5,6]. There were no changes in the DNA sequence of the in the inactive form of the X chromosome, so how did the X chromosome become silenced? Riggs hypothesized that the X chromosome was silenced by DNA methylation, a covalent modification to the DNA, a type of epigenetic mark [7]. Doskočil and Storm found an inheritance mechanism of this epigenetic mark through DNA replication [8]. Patterns of DNA methylation marks on the parent strand were copied and then passed down to the daughter strands (Figure 3).

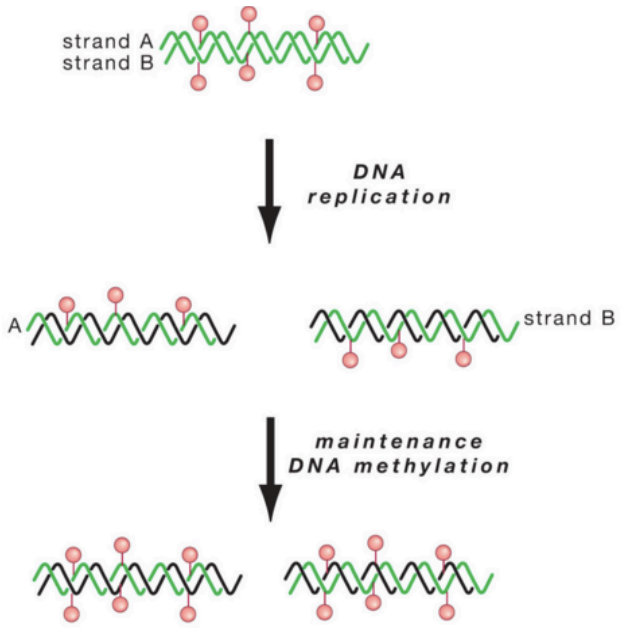


Figure 3. Maintaining DNA Methylation. During replication one methylated strand pairs with an unmethylated strand. DNA methyltransferase recognizes the hemimethylated DNA and methylates the new strand in order to maintain the methylation pattern. [1]

The inactivation of the X (Xi) chromosome is a random event to initiate a dosage compensation [6]. An X inactivation center (XIC) located on the X chromosome has a large non-coding RNA, X-inactive specific transcript (Xist) in *cis* with the chromosome that is responsible for the inactivation [9]. There is an upregulation of Xist RNA covering the X chromosome to initiate silencing [10]. Xist promotes the recruitment of PRC1 (polycomb repressive complex) and PRC2 which have catalytic activity towards chromatin structure [11]. Histone 2A Lys119 ubiquitylation (H2AK119ub1) and histone 3 Lys27 trimethylation (H3K27me3) are mediated by PRC1 and PRC2 respectively on Xi [12,13]. X chromosome inactivation is associated with the methylation of H3-K27 by the Eed-Ezh2 complex [12,13].

In 1942, Conrad Waddington created the term "epigenetics". He described an "epigenetic landscape" on the basis of how the genes interact with their environment during development to form a specific phenotype; the end result is not completely determined by parental genome. The classic image that he describes is a marble rolling down to the lowest elevation which represents how the environmental landscape can influence gene expression [14] (Figure 4).

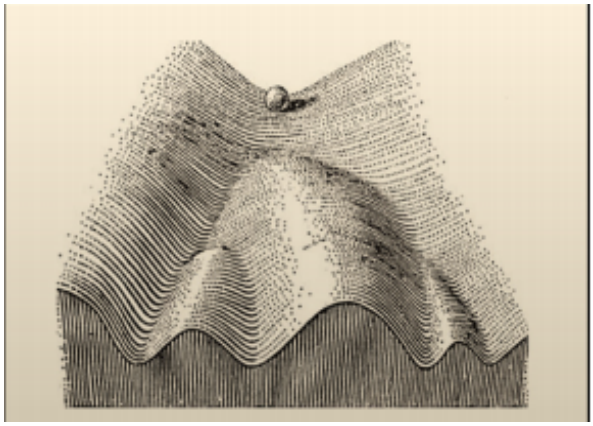


Figure 4. Waddington's Epigenetic Landscape. A metaphor for cellular decision making during development of a marble rolling downhill. [14]

1.1.2. The Difference between Genetics and Epigenetics

We are more than the sum of our genes [15].
You can inherit something beyond the DNA sequence. That's where the real excitement in genetics is now [16].

Epigenetics is reversible, stable, and plastic. In contrast, DNA is non-reversible, static, and heritable. It is thought that epigenetics alters transcription, dictating which proteins are made based on modifications to the chromatin structures (Figure 5). Chromatin is comprised of tightly bound DNA wrapped around eight histone protein

cores to form a nucleosome. Depending on how tightly the the chromatin is wrapped, it can form euchromatin or heterochromatin. The former resembles “beads on string” while the latter is very highly condensed.

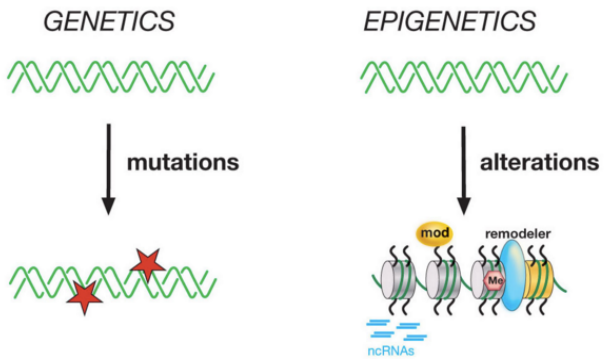


Figure 5. Genetics vs. Epigenetics. Genetics is heritable and mutations can occur (red stars). Epigenetics alters the chromatin structure by histone modification (mod), chromosome remodeling (remodeler), DNA methylation (Me), non coding RNAs (blue), and histone variant composition (yellow). [1]

DNA is comprised of four nucleic bases - adenine, thymine, cytosine, and guanine with each base attached to a deoxyribose and a phosphate group. One of the most well-known epigenetic process is DNA methylation of cytosine in CpG islands that are in promoter rich areas. Additionally, another well-known epigenetic process is posttranslational modification of histone proteins [17].

The nucleosomes consist of an octamer with two sets of H2A, H2B, H3, and H4 [18]. Amino acids located on the tail ends of the histone proteins can undergo posttranslational modification by acetylation, methylation or other covalent modifications. Depending on the modifications made, the structure and recruitment of

DNA binding proteins can loosen (euchromatin) or tighten (heterochromatin) the chromatin resulting in repression or activation of genes respectively [19].

Currently, “epigenetics [...], is at the epicenter of modern medicine because it can help to explain the relationship between an individual's genetic background, the environment, aging, and disease” [20]. Each individual has their own epigenome dependent on their family history and lifestyle environmental exposures such as drugs, diet, and exercise. All these factors need to be taken into account during treatment or postulating a certain condition in the medical field. As such, epigenetics is hypothesized to be the basis of many developmental disabilities including fetal alcohol syndrome, Beckwith-Wiedemann syndrome, Rhett syndrome, autism, and Fragile X syndrome [21,22]. The study of epigenetics will be crucial for the development of new medical practices and therapies.

1.2 Methylation

1.2.1 Process of DNA Methylation

DNA methylation is the first discovered heritable mechanism to influence gene expression [23]. DNA methylation is the addition of a methyl group (-CH3) on the fifth carbon of a cytosine nucleotide to form 5-methylcytosine. These modified bases are often found in CpG islands by a family of enzymes called DNA methyltransferases (DNMT) (Figure 6). The methylation groups are added to the major grooves of DNA. This addition affects the protein interaction in prokaryotes and is probably analogous in eukaryotes [23].

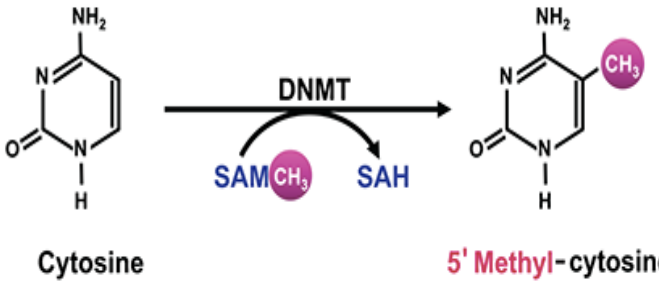


Figure 6. DNA Methylation. The addition of a methyl group by DNA methyltransferase with the aid of S-adenosylmethionine (SAM) which is converted to S-adenosylhomocysteine. (SAH).[24]

The genome contains many "CpG islands [that] are short, dispersed regions" of methylated DNA [25]. About 70% of CpG islands are methylated in the animal cell [26]. In 1992, Larsen identified 240 CpG islands associated with genes after analyzing a total of 375 genes. Almost all the identified CpG islands covered some part of exon 1; therefore, CpG islands were viewed as a good tool for identifying genes [25].

When DNA methylation occurs, there are structure and energy signatures that appear in the DNA. For example, in the major groove of DNA, there is a steric change from the hydrophobic methyl group that affects the conformation of DNA and impacts the accessibility to proteins. The stability of the helices increases due to lower energy from the cytosine methylation, and increases the melting temperature (Tm) of the DNA. Also, the addition of the methyl group alters the local charge of the major groove by increasing electrostatic interaction with positively charged groups [27].

The noncoding and repetitive regions of DNA can be found by looking for CpG densely patterns of methylation. In higher eukaryotes, with an increase in noncoding and repetitive regions, there is a corresponding increase in DNA methylation. (Figure 7). DNA methylation protects the genome by silencing nucleic acids of exogenous origin, such as transposable elements (e.g. ones observed in maize by McClintock) and repetitive sequences [1][4].

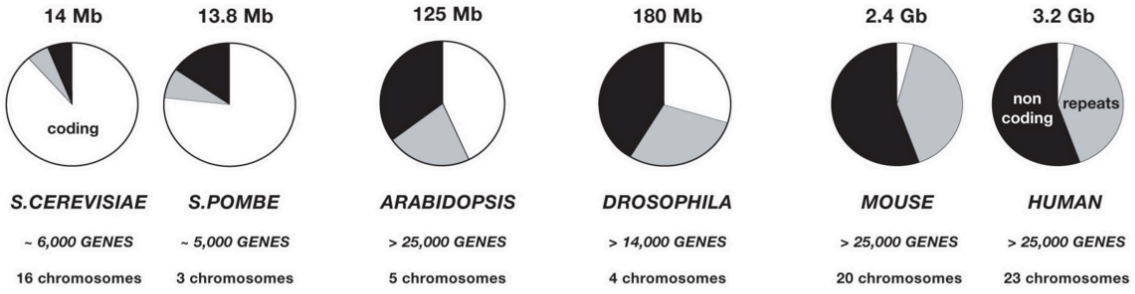


Figure 7. Genome Organization Based on Organism. The non-coding (black) and repetitive DNA (grey) increases with increasing more complex organism. The white portion is the amount of coding genes in each organism.[1]

When DNA is methylated, proteins cannot easily access the DNA, making transcription difficult. Therefore, by interfering with transcription factors, DNA methylation is associated with transcriptional silencing [26,28]. Conversely, "active" genes are associated with demethylation because RNA polymerase II can easily transcribe DNA. For reference, only 20-30% of genes that are expressed are methylated [26].

1.2.2 Regulation of DNA Methylation

In mammals, the family of enzymes termed the DNA methyltransferases or DNMTs regulate DNA methylations. This family is composed of DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L. DNMT1's role is to maintain methylation [29]. Li showed when DNMT1 is deactivated, it resulted in a global loss of methylation, growth reduction, and lethality of the embryo. The mutant embryos were 1/8th the size of the wildtype or heterozygous embryos, and most mutant embryos had an open anterior neuropore and no visible forelimb buds [30]. Schaefer found that DNMT2 methylates several tRNAs and protects tRNA from ribonuclease cleavage [31]. When DNMT2 was knocked out in mice, there was a delayed endochondral ossification of the long bones, and a reduction of hematopoietic stem and progenitor cells at day 8 postnatal [32]. There are 3 homologous genes of DNMT3 - DNMT3a, DNMT3b and DNMT3L. De novo methylation by DNMT3a and DNMT3b are essential for development [33]. Knockout DNMT3a mice were developed at term and appeared normal compared to the wild type. However, the knockout mice were eventually stunted and died at four weeks of age [33]. The second form, DNMT3b, is associated with Immunodeficiency, Centromere

Instability and Facial Anomalies (ICF) syndrome in humans[34]. DNMT3b knockout mice did not make it to term, but observations during gestation showed there were several defects including growth retardation and neural tube defects[33]. Lastly, DNMT3L is a regulator of DNA methylation associated with genomic imprinting and germ cells. In knockout mice, DNMT3L, females failed to maintain maternal imprints and resulted in no live offspring [35]. Whereas in knockout males, spermatocytes did not complete meiosis and resulted in sterility [36]. DNMT3L expressed during gametogenesis interacts with DNMT3a and stimulates maternal methylation of Snrpn and Igf2r/AirI [37]. DNMT3L has no catalytic activity, but encodes a protein needed to stimulate DNMT3a and DNMT3b to regulate genomic imprinting [35].

1.2.3 DNA Methylation Pathology

Rett Syndrome is another result of misregulation of methyl binding protein. Rett syndrome is a neurological condition on the X chromosome. However, because methyl CpG binding proteins read methylation, if there is a mutation on the methyl-CpG binding protein 2 (MeCP2) in humans, it can lead to Rett syndrome. MeCp2 binds to methylated CpG to the adjacent adenine and thymine to repress transcription. Since females have an inactivated X chromosome, they will exhibit the mutant or wild type gene. Females affected by Rett syndrome will show neurological signs 6-18 months after birth. However, males that are hemizygous for Rett syndrome will not survive [1].

Methylation may also be linked to cancer [1]. There are three ways DNA methylation is correlated with cancer: hypomethylation of the cancer genome, hypermethylation of tumor suppressor genes, and direct mutagenesis[38–40]. Abnormal

methylation on CpG islands in genes is also associated with cancer. There are more hypomethylated DNA in tumors compared to non-tumor genes [41,42]. In 1988, Cooper and Youssoufian stated 35% of human genetic disorders were caused by point mutations in the CpG dinucleotides, and 90% of those were from cytosine to thymine [43] (Figure 8).

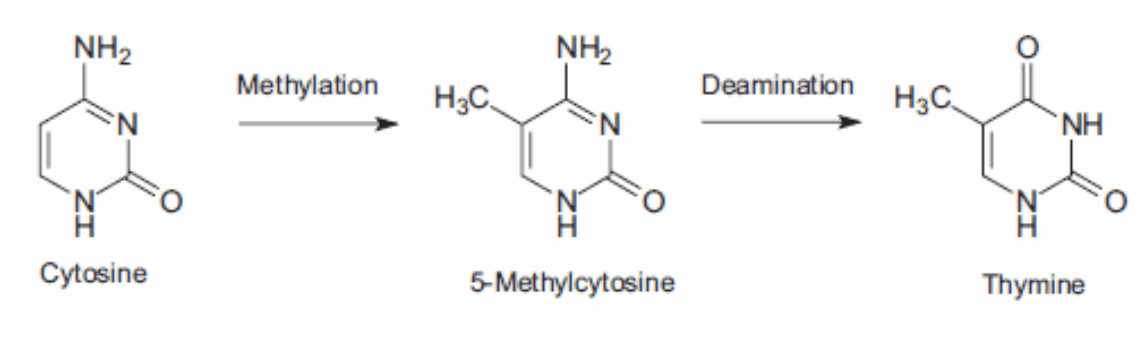


Figure 8. Conversion of 5'Methylcytosine to Thymine. Through deamination 5'methylcytosine is converted to thymine creating a point mutation. [44]

1.3 Imprinting

1.3.1 Imprinting Mechanism

In 1984, McGrath and Solter demonstrated that both the maternal and paternal genomes are required to complete embryogenesis in order to produce offspring. In their experiment, they used two female pronuclei (biparental gynogenone) or two male pronuclei (biparental androgenone) to produce a diploid mouse embryo. Their results showed that the biparental gynogenone/androgenone diploid embryo did not complete embryogenesis compared to the control embryos. "While both parental sexes contribute equivalent nuclear information to the zygote, this genetic information is not necessarily functionally equivalent." This result indicated that both the maternal and paternal genomes are needed during embryogenesis [45].

There are around 100 imprinted genes in mammals [46]. The first imprinted genes were discovered in 1991 and were all located in the same imprinting control region (ICR). The first imprinted gene discovered was Insulin-like growth factor type 2 receptor (Igf2r), a maternally expressed gene. A deletion of this maternal allele resulted in death for the embryo at E15 [47]. A short time later a paternally expressed gene, Insulin-like growth factor 2 (Igf2) was discovered [48]. The discovery of Igf2 as an imprinted gene, showed that it has a major role in fetal growth during gestation. Lastly, the maternally expressed gene H19 was discovered by Bartolomei, a few months after Igf2. It encodes for noncoding RNA [49].

Noncoding RNA are highly important in regulating genomic imprinting. Many imprinting clusters contain noncoding RNA and protein coding genes which are

reciprocally expressed such as H19,Igf2, Igf2r/Air, Dlk/Gtl2, and Kcnq1/Kcnq1ot1 [50–52]. It is hypothesized that RNA may recruit epigenetic factors, such as PRC2, that regulate the imprinted expression of the coding genes [53,54].

Imprinted control regions (ICR) are usually 1Mb clusters that contain both maternal and paternal imprinted genes and at least one non coding RNA [46]. The regulation of imprinted genes is due to DNA methylation. The CpG islands found at the promoters of imprinted genes are mostly methylated and repressed whereas the expressed gene is unmethylated. These regions are called differentially methylated regions (DMR) [46].

DNA methylation is best studied as a regulatory mechanism in genes regulated by genomic imprinting [55]. Genomic imprinting occurs when specific genes are expressed by only one parent's allele. For example, if a gene is maternally imprinted, the maternal allele has less expression while the paternal allele is predominantly expressed (Figure 9). As shown by McGrath, both parental genomes are required for the development of a fetus [45]. Genomic imprinting is controlled by DNA and histone methylation [55].

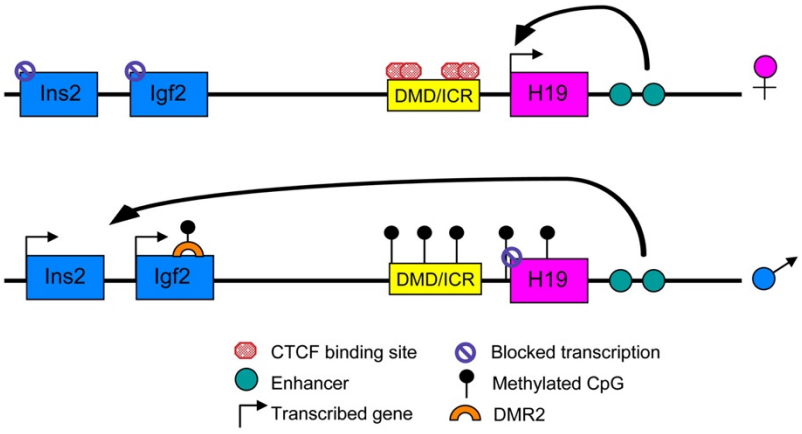


Figure 9. Genomic Imprinting. Maternal allele expression of H19 with the H19 paternal allele imprinted. The Igf2 paternal allele is expressed while the maternal allele of Igf2 is imprinted.[56]

There are two critical stages of DNA demethylation in mammalian reproduction. In the first stage, primordial germ cells undergo global demethylation, which includes the erasure of parental imprinted methylation [57]. Once the gametocyte is fully developed and mature, de novo methylation is already reestablished in a parent of origin specific pattern (Figure 10). The second stage of demethylation (reprogramming) occurs only with the non-imprinted genes and is local only up to the blastocyst. There is an active paternal demethylation of somatic genes in the pronucleus while a passive maternal demethylation of somatic genes in the preimplantation embryo [58] (Figure 10). The genome resumes de novo remethylation by DNA methyltransferase after implantation [59]. The imprinted genes retain their methylation marks during this stage of the reprogramming period, which allows for monoallelic expression [60] (Figure 10).

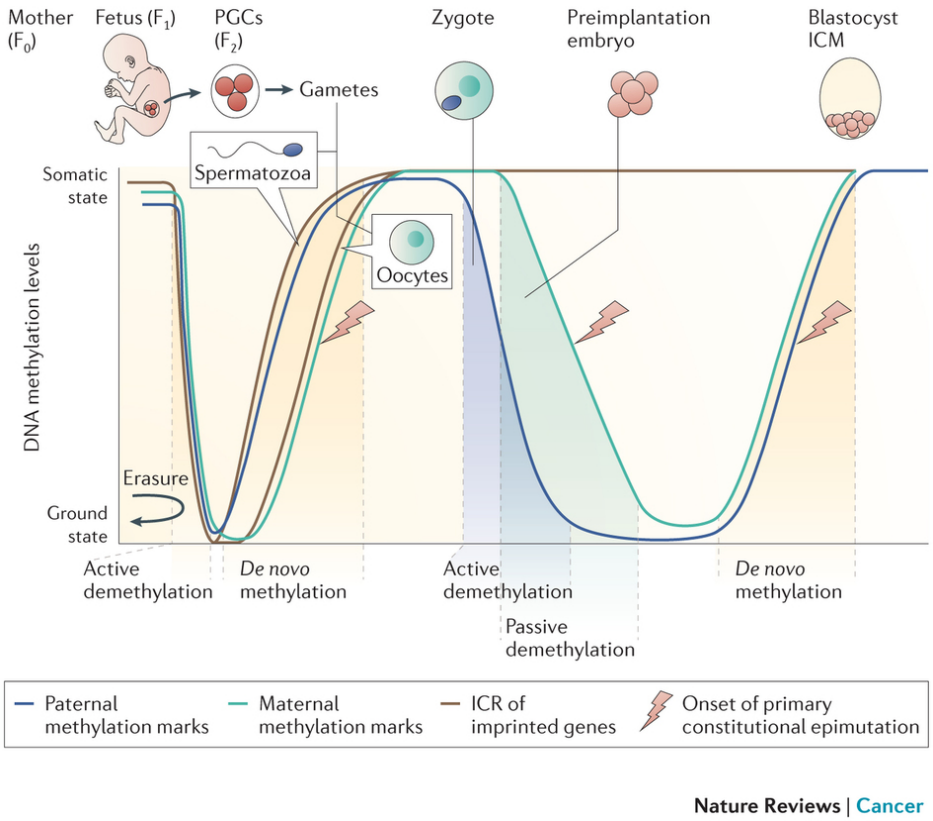


Figure 10. Methylation Reprogramming. Paternal methylation marks are indicated with the blue line, maternal methylation marks are indicated with the green marks, while the red line indicates the methylation of genes in the imprinting control regions. The orange lightning bolt indicates the most places in reprogramming that epimutation can occur.[61]

1.3.2 Faulty Expression of Imprinted Genes

Disruptions in imprinted genes expression are a method to detect environmental perturbations on the epigenome. With only one allele expressed, depending on the parental origin, it is easier to look at the environmental effects on the gene of the gametes through inheritance of imprinted patterns of gene expression [62]. Environmental factors, such alcohol and BPA, may disrupt regulation or imprinting. Assisted reproductive technologies (ART) may also disrupt imprinting in ART, the

culture media may also influence imprinted gene expression of H19. When embryos were cultured in Whitten's media until the blastocyst stage, the normally silent paternal allele of H19 was expressed [63]. H19 and Snrpn also showed biallelic expression in the placenta, but not in the embryo. The embryo exhibited reduced methylation at the ICR of Snrpn and H19 [64]. Haycock and Ramsay demonstrated that, when pregnant dams were exposed to alcohol, methylation decreased in the paternal alleles in the placenta [65]. Dams exposed to BPA in the late stages of oocyte development deregulated imprinting in the placenta, most notably in Snrpn, Ube3a, Igf2, Kcnq1ot1, Cdkn1c, and Ascl2. Additionally, a genome-wide methylation analysis found the placenta had reduced methylation compared to the embryo [66].

When these imprinted genes are disturbed, the results can be devastating. Changes in imprinting are responsible for Beckwith-Wiedemann syndrome, Prader-Willi syndrome, Angelman syndrome, and Silver-Russell syndrome.

Angelman syndrome (AS) is named after Harry Angelman. He identified the following characteristics in AS children: mental retardation, speech impairment, ataxia, and microcephaly (Figure 11a). AS is thought to be caused by a deletion in chromosome 15 where the maternal gene is usually expressed. One in every 15,000 to 20,000 children are diagnosed with Angelman syndrome [67].

In the same region that affects AS, there is another imprinted gene that is maternally imprinted with the paternal allele being expressed. When the paternal expression is lost, it can lead to Prader-Willi syndrome (PWS). Langdon-Down who first described Down syndrome also described the characteristics of Prader-Willi syndrome

before Prader, Labhart, and Willi [68]. PWS is characterized by childhood obesity, lack of muscle strength, and cognitive impairment[67,69] (Figure 11b). The estimated cases of PWS in the world are 1 in 10,000-30,000 [70].

Bruce Beckwith and Hans-Rudolf Wiedemann discovered a new syndrome called exomphalos-macroglossia-gigantism syndrome (EMG) in 1963 that later became known as Beckwith-Wiedemann syndrome (BWS) [71]. BWS children have clinical features of microcephaly, macroglossia, neonatal hypoglycemia, and exomphalos [46] (Figure 11c). The most frequent cause of Beckwith-Wiedemann Syndrome is the loss of the maternal allele at region 11p15.5 resulting in a double expression of the paternal allele[72]. This is also called uniparental paternal disomy [73]. It is estimated that BWS affects 1 in 14,000 children[72].

In 1969, Tanner and Ham are responsible for terms “Silver dwarf” and “Russell dwarf” that are known collectively now as Silver–Russell syndrome (SRS) [74]. SRS children have characteristics of intrauterine and postnatal growth retardation, triangular face and learning disabilities[46] (Figure 11d). The most common cause of SRS is associated with H19 and IGF2 in the imprinting region of 11p15 [75]. One in 75,000 - 100,000 people are affected with SRS [76].

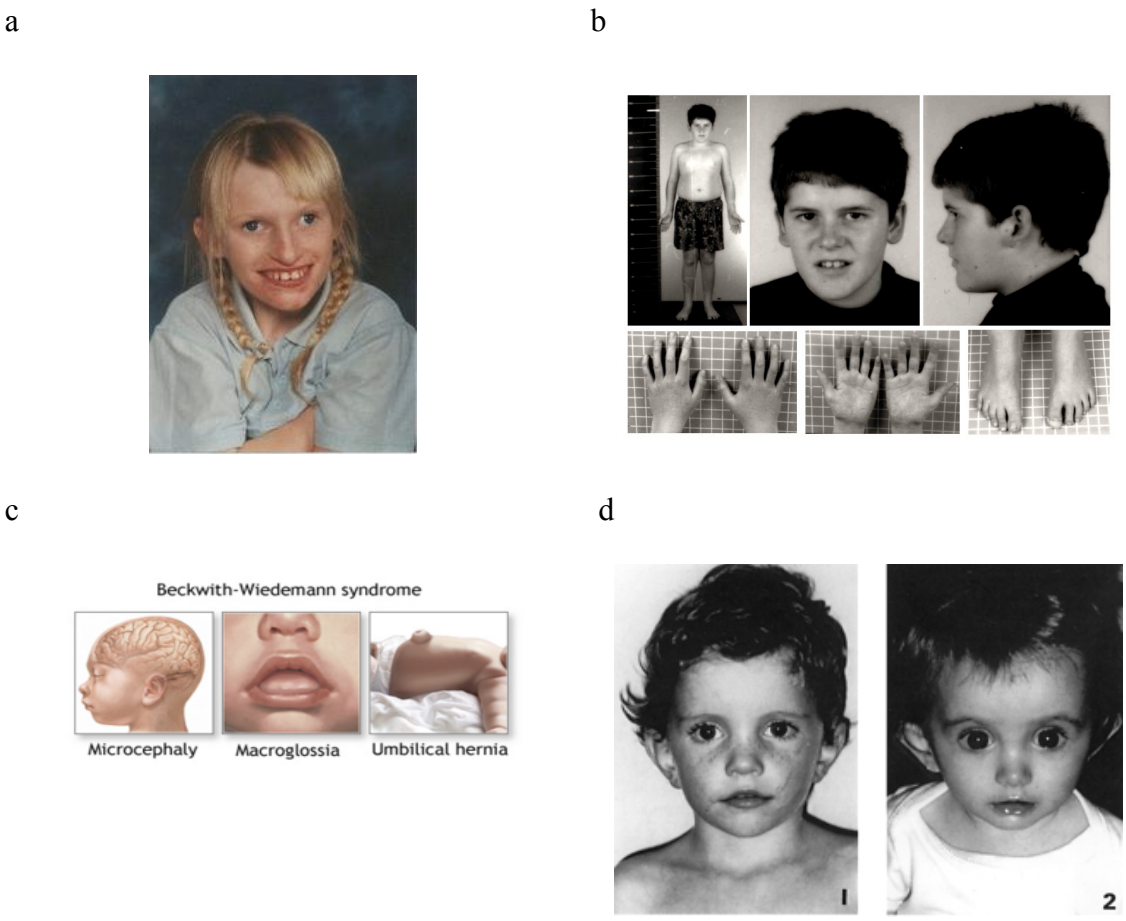


Figure 11. Common Imprinting Disorders. (a) Angelman Syndrome, (b), Prader Willi-Syndrome, (c) Beckwith-Wiedemann Syndrome, and (d) Silver-Russell Syndrome. [77–80]

1.4 Spermatogenesis

1.4.1 The Making of Sperm

Spermatogenesis is the process of forming spermatids from undifferentiated male germ cells - spermatogonia - in the seminiferous tubules of the male testes. Spermatogenesis has three main phases: proliferation, meiosis, and differentiation (Figure 12). During the proliferation stage, spermatogonia proliferate to produce more spermatogonia. The meiotic stage occurs when the primary spermatocytes undergoing mitotic division to produce haploid secondary spermatocytes. This stage insures an increase in genetic diversity. The final stage, differentiation is when the spermatid undergoes morphological changes to become spermatozoa [81].

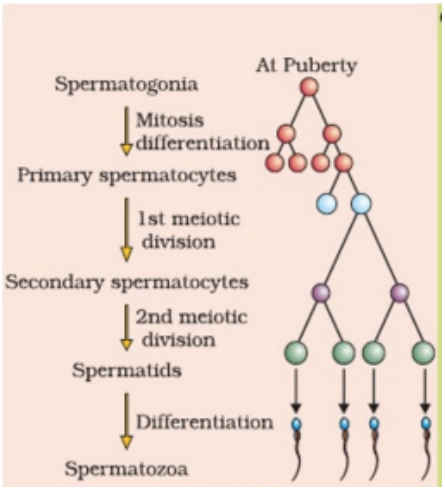


Figure 12. Phases of Spermatogenesis. Three phases of spermatogenesis are proliferation, mitosis, and meiosis.[82]

Like all mammalian species, mice have a spermatogenesis cycle specific to their species. Their meiotic cycle begins in the fetus when the seminiferous tubules form, which contain Sertoli cells and germ cells. Meiosis begins at during the first week post birth, and the first spermatids are made by day 22-24. Complete sperm are formed in the mouse by day 28, but quantitatively not enough sperm for fertilization[83].

There are 12 stages and four cycles ending in 33 days to produce mouse spermatids in the seminiferous tubules (Figure 13). Each cycle takes 8.45 days and each cycle has 12 stages that occur in the seminiferous tubules. After 33 days, spermatid maturation occurs in epididymis[84].

The final product is a 65mm spermatozoa comprised in three sections: head, midpiece, and tail. The head is where the genetic material resides, a haploid nucleus and acrosome head resides, and midpiece is where the mitochondria reside. The tail is where the microtubules that constitute the flagellum reside, which enables the motility of the sperm[81].

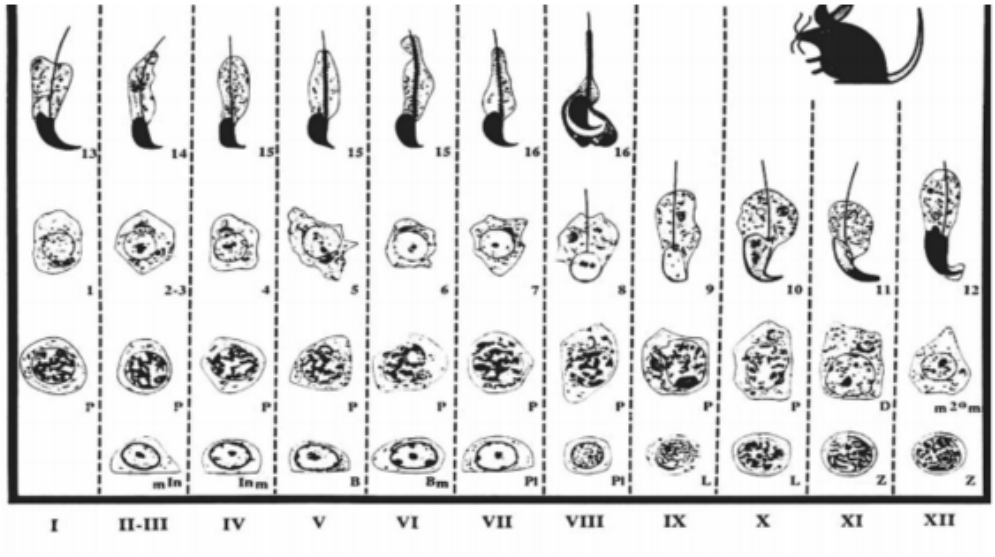


Figure 13. Mouse Spermatogenesis. The 12 stages involved in spermatogenesis to produce spermatozoa in the seminiferous epithelium. [85]

1.4.2 When Problems Arise in Spermatogenesis

When spermatogenesis is disrupted or incomplete, it can lead to problems in fertility and the development of offspring. Factors that can influence spermatogenesis include radiation, folate, obesity, age and alcohol.

Some men who have undergone chemotherapy or radiation can become infertile. The germinal epithelium is sensitive to radiation which can change the spermatogonia. The amount of radiation can be as little as 0.2 Gray (unit of absorbed radiation)[86]. If the spermatogonia are damaged, the process of proliferation is halted during spermatogenesis.

As men age, there is an inverse relationship with sperm morphology. Sperm morphology decreases 0.9% each year, totaling a 18% decrease in a male who is 50 years of age in comparison to a 30-year-old man [87]. Both morphology and motility

change with age. Occurrences of microcephalic heads and coiled tails increase as well with with age [88]. According to Auger, there is a 0.6% decrease each year in motile sperm with a 12% decrease in motility in a 50 year old man in comparison to a 30 year old man [87].

In 2013, Lambrot gave male mice a low folate diet [89]. The offspring sired by experimental males were born with more birth defects such as craniofacial and musculoskeletal malformations. A genome-wide DNA methylation analysis on the sperm found different methylation patterns on the genes associated with autism, schizophrenia, diabetes, and cancer.

Most recently, Donkin profiled the epigenome of lean and obese men. The patterns of noncoding RNA abundance and DNA methylation were quite different, but the histone positioning was similar. His study found that men who were obese and had undergone surgically induced weight loss showed a drastic change in the DNA methylation in their sperm [90]. Ergo, there is a correlation between environmental factors present in the father's lifestyle that will affect the gene expression of the sperm.

Additionally, male consumption of ethanol can lead to decreased semen volume, sperm count, motility, and abnormal sperm morphologically [91]. Finally, male mice were chronically exposed to differing amounts of ethanol, and those exposed the most to ethanol had the most decrease in sperm motility [92]. Furthermore, sperm from alcoholic mice showed not only a decrease in quality, but in fertilization capability as well [93].

1.5 Parental Contributions to Development

1.5.1 Parental Nutrition

Couples planning their pregnancy are encouraged to lead healthy lifestyles through diet and exercise. During a prenatal visit, obstetricians will ask questions about the women's smoking, alcohol, medication, and family history. The father, typically, is rarely asked about his lifestyle except for family history. As shown below, recent research suggests the father's lifestyle is an important factor in prenatal development and indicates the father's inheritance is more than DNA.

For example, if paternal grandfathers experienced famine, then their grandchildren were more likely to be obese [94]. Over thirty years ago, David Barker found a correlation between low birth weights and ischemic heart disease, and poor nutrition and insufficient oxygen during gestation [95,96]. These effects are now called "Barker's Hypothesis", and encompasses the study of developmental origins of health and disease.

Another recent example shows that offspring sired from males fed a low protein high fat diet had an increased expression levels of hepatic genes associated with lipid and biosynthesis [97]. In addition, fathers exposed to a high-fat diet had female offspring with elevated b-cell dysfunction, which is a major precursor to type 2 diabetes [98]. Fathers who exercised regularly had offspring with altered levels of energy expenditure and insulin resistances [99].

Stress is another environmental factor that can cause epigenetic triggers. Male mice exposed to stress showed an increase expression with nine microRNAs. The

increase is linked with a reduction in the hypothalamic-pituitary-adrenal (HPA) axis stress responsivity. Their offspring had a decrease in stress responsivity and increased expression of glucocorticoid response genes [100].

Gene regulation can also be influenced by drug usage. When male rats self-administered cocaine, the male offspring had an increase in mRNA and protein expression of corticotrophin-releasing factor receptor 2 in the hippocampus. Their male offspring showed an increase in anxious behavior [101]. Another trial by Lee in 2009, showed that there was an increase in acute lymphoblastic leukemia in offspring whose fathers smoked at home due to the absence of a haplotype of cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) [102].

1.5.2 A More Focused Look on Fetal Alcohol Syndrome

Alcohol, a known teratogen, is found to affect the development of the fetus by altering gene expression [103]. It is also known to disrupt the regulation of DNA methylation in the genome [104].

Fetal Alcohol Syndrome (FAS) is a common disorder affecting six to nine per 1,000 live births [105]. The effects of FAS are devastating, characterized by growth and mental retardation, neural and craniofacial abnormalities, and decreased birth weight (Figure 14). Until recently, only the maternal contribution to FAS has been studied. However, due to patterns of imprinted gene expression, paternally inherited genes are a major driver of placental development; therefore, the effects of paternal alcoholism must be studied in relation to the observed FAS growth phenotypes [59]. As discussed in the previous section, environmental factors present in a male's lifestyle

influence spermatogenesis through epigenetic mechanisms, which in turn can cause congenital defects. Fetal Alcohol Syndrome may be one such effect.

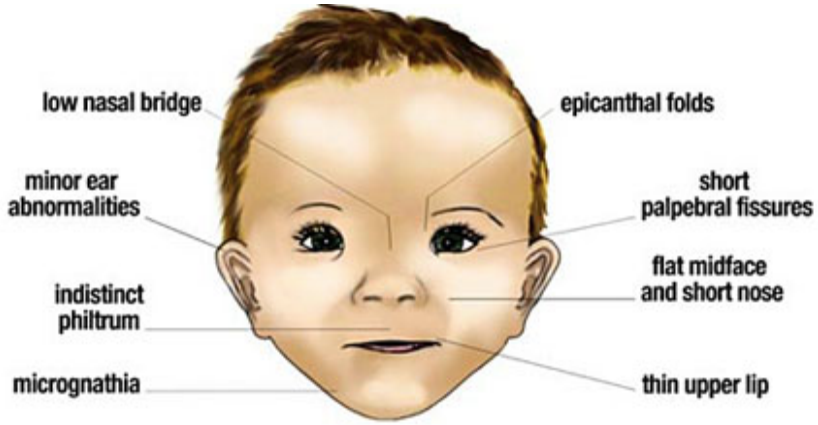


Figure 14. Fetal Alcohol Syndrome Characteristics. Low nasal bridge, ear abnormalities, smooth ridge between upper lip and nose, small jaw, thin upper lip, flat midface, short nose, small opening between eyelids, and skin fold of the upper eyelid covering the inner corner of the eye.[106]

Until recently, FAS was thought to be solely caused by maternal alcoholism. However, growing evidence suggests fathers may also contribute FAS. One in ten adults in the U.S. meet the criteria for alcohol dependence [107]. Of these, men are more likely to drink excessively during their young adulthood years (18-24) and have 12.5 binge drinking episodes per year, far exceeding women's reported levels of 2.7 episodes per year [107,108]. According to the National Institute on Alcohol Abuse and Alcoholism a binge drinking episode is defined as the consumption of five or more drinks within a two-hour period, to exceed a blood alcohol content level of 80 mg/dL [109]. Due to this

magnitude of paternal alcoholism, and prior evidence suggesting the influence of male lifestyles upon their offspring, the effects on reproduction must be examined.

There has been little research on chronic exposure of alcohol of a father and the effects of offspring. Previous research has found children sired from alcoholic fathers have reduced birth weight, decreased cognitive ability, and increased hyperactivity [110–112]. Additionally, offspring of chronically exposed alcoholic mice exhibit hearing loss [92]. When male mice were exposed to alcohol, their offspring were shown to have decreased litter size, birth weight, and decreased cognitive ability [113–115]. These observations have been shown in paternal mice that were drinking while mating as well as after withdrawal periods prior to mating [116].

The molecular effects of males chronically exposed to alcohol have has very little attention. One study observed the methylation of spermatozoa and cerebral cortices of offspring sired from male mice chronically exposed to ethanol. However, this study used Kumming (Km) mice that were given ethanol every 2 days for four weeks. Spermatids in male mice take 33 days to form (Subheading 1.4.1). Liang did not wait for the male mice to be exposed to alcohol for at least one cycle of spermatogenesis before mating with untreated females and failed to clear the unexposed premeiotic sperm. The methylation patterns of imprinted maternally expressed transcript non-protein coding (H19) and paternally expressed 3 (Peg3) in spermatozoa showed a significant decrease and increase respectively. Three CpG islands of Peg3 in the cerebral cortices had a significant increase that mimicked the CpG islands of Peg3 in the spermatozoa[92] Their result does not indicate a strong correlation of methylation between the spermatozoa and

cerebral cortex of Peg3. There are eleven CpG islands in PEG3 and only 27% of the CpG islands in the cerebral cortex mimicked the sperm CpG island methylation in Peg3. However, this study did indicate that exposure to alcohol alters methylation patterns in imprinted genes in spermatogonia and could give rise to developmental disorders such as FAS [92].

Given the body of presented evidence, there is likely to be a correlation between male alcohol consumption and congenital birth defects such as Fetal Alcohol Syndrome through an epigenetic mechanism. The hypothesis of this work is the offspring from males chronically exposed to alcohol will exhibit some of the characteristics of Fetal Alcohol Syndrome. Chronic male exposure to alcohol may have a role in FAS but to date this has not been rigorously investigated. We suspect that the offspring will have smaller fetal weights, placentas, gestational sacs, and longitudinal and sagittal lengths. The sperm from the chronically exposed males will have a decreased motility, and the paternal genomic imprints will be altered. We have elected to focus our studies on several key imprinted genes, as these are the ones best supported by the literature, we have validated reagents to do so, and their misregulation often serves as a "canary in the coal mine" for other epigenetic abnormalities [117]. Further, Peg3 loss of imprinting influences placental growth but exhibits normal morphology, while Igf2 loss of imprinting is associated with errors in morphological patterning [55,118]. Thus these genes have well-characterized links to fetal and placental growth parameters, which we will be measuring.

CHAPTER II

MATERIALS AND METHODS

Experimental mice were maintained on a 12-hour light/dark cycle where ethanol was made available during the initial four hours of the dark cycle. This "drinking in the dark" paradigm is similar to the one first described by Rhodes et al., where ethanol was introduced early in the dark cycle by replacing the water bottles with a 10% ethanol solution. This model was selected due to its demonstrated ability to produce high blood ethanol concentrations, which are similar to those obtained in other models of chronic alcohol exposure, but without the stress of excessive mouse handling [119]. In our experiments, male mice, B6(Cast7), were treated with 10% ethanol solution with 0.066%(w/v) saccharin for 70 days, while the control male mice, B6(Cast7), were exposed to 0.066% (w/v) saccharin ad libitum [120]. C57BL/6J and B6(Cast7) mouse strains were chosen to easily observe allelic patterns and transcription. The B6(Cast7) on portions of chromosome 7 and 12 from *Mus musculus castaneus* (CAST has at least 30 imprinted genes and 5 imprinting domains) [121]. These traits make examining maternal and paternal alleles easier.

The experimental mice were initially treated with 5% ethanol solution for a week to introduce ethanol to them. After one week, the ethanol was increased to 10%. The 70 day duration included 18 days of the formative growth phase within spermatogenesis [122]. There was one mating at day 35 to clear the preformed sperm with non-experimental C57BL/6J dams for all male mice (experimental and control). Because the mice were exposed to ethanol for 70 days, and mated midway at day 35, this ensured all

post meiotic sperm was exposed to ethanol (Figure 15). All experiments done under AUP 2014-0087 and approved by Texas A&M University IACUC.

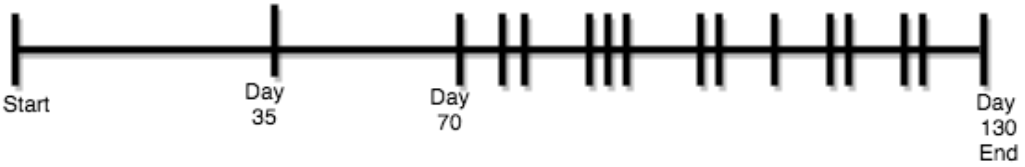


Figure 15. Times Mice Were Bred. The vertical hash marks indicate when males were off ethanol for a single night for breedings. The maximum time off ethanol was 3 consecutive days (Not drawn to scale.)

Four blood samples of 20μL were collected throughout the duration of ethanol exposure to measure their blood ethanol content from the lateral saphenous vein and measured with gas chromatography[120]. Using a Varian 3900 Gas Chromatograph with 8400 Autosampler the ethanol(mg/dl) of each blood sample was taken. Twenty microliters of blood were dispensed into a vial with 200 μL of a solution of 70% perchloric acid and 100% n-propanol. The final concentration of the solution was 0.6 N perchloric acid and 4 mM n-propanol. The vial containing the blood and solution was kept at room temperature overnight and run the following day.

After three successful matings that led to pregnancies, male mice were sacrificed with carbon dioxide and cervical dislocation. Human tubal fluid (HTF) media (Life Global Group, GMHT-100) was supplemented with 4mg/ml of BSA fraction V (Sigma Aldrich, Cat. No. A5611 or A9647) and 1μl/ml of Gentamicin (Gibco 15750-060) was

prepared three hours before males were sacrificed. One dish was prepared (35 x 10 mm Petri) for each sample with 1 ml of HTF media and covered with mineral oil, the dish set in an incubator at 37°C in a 5% CO_2, 5%O_2, 90% N_2 atmosphere. The remaining HTF medium was stored in the same incubator for three hours.

The male mice were placed on their back and sprayed with 70% ethanol. A cut was made into the skin to expose the peritoneum and body cavity. The skin was pulled away to expose the testis and epididymis to locate the vas deferens. Connective tissue was dissected away from the vas deferens to remove as much of the fat and bloods vessels that surround it. A cut was made 0.5cm away from the epididymis, and the vas deferens was placed into a petri dish that contained warmed handling medium (PBS). The vas deferens was then placed into the petri dish containing the mouse fertilization media from the incubator. Forceps were used to remove the sperm through the cut end and three or four holes were punctured into the epididymis to facilitate the release of sperm. The dish was then placed back into the incubator for thirty minutes.

The concentrated sperm from the vas deferens and the whole epididymis were placed in a 5mL polystyrene round bottom tube (BD Falcon, Cat. No. 353058) at a 45° angle in the incubator for 1 hour. This facilitated the live sperm to swim up from the debris and dead sperm. Then 1.5 ml were removed from the top of the round bottom tube and placed into 1.5 ml tube. Sperm were diluted to a 1:1 dilution factor with millipore water and counted using a hemacytometer.

After 70 days of regular drinking, the male mice were mated with non-experimental dams, C57BL/6J. The pregnant dams were sacrificed at E14.5 by CO2 and

cervical dislocation. On E14.5, the maternal decidua was easily removed from the fetal interface and fetal tissues (placenta, brain, and liver) and measurements (dam, fetus, gestational sac, placental weights and longitudinal sagittal length) were taken. The placenta was easily removed from the fetus and cut into four equal parts. The liver was dissected out of the fetus, and the brain was cut into equal halves. The remaining fetal tissues were kept and used for sexing the fetus. The fetuses' location in the uterine horn (left- L or right - R) was recorded. The tissues were dissected using standard dissections tools and the weights were obtained using a scale. The longitudinal and sagittal length was measured with calipers.

RNA was isolated from placental tissue using the RNeasy Mini Plus Kit (Invitrogen, Cat. No. 74134) following the manufacturer's guidelines. The placental tissue DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Cat. No. 69504) following the manufacturer's guidelines. Both the RNA and DNA were stored at -80°C until further use.

RNA sequencing was performed on an Illumina HiSeq 2500 from the MIT Whitehead Institute to examine the individual transcriptome of eight samples. The eight samples were comprised of two females and two males from ethanol exposed sires and two females and two males sired from control males.

Three samples from the control mice sperm and three samples from ethanol exposed mice sperm used to determine the methylation of the CpG sites in the genome. The sperm DNA was sent to Epigentek for enhanced methyl-seq. The samples were subjected to enzymatic digestion (MSP1 + TaqI), library preparation, bisulfite

conversion, Bioanalyzer QC, KAPA library quantification, multiplex NGS on an Illumina HiSeq2500 and advanced data analysis.

Gene expression was done on Kcnq1ot1, H19, Igf2r, Kcnq1, Ascl2, Gtl2, Peg3, and Kcnq1 that are imprinted genes in the placenta and have been associated with placental developmental defects [55,118] . Three of which are paternally expressed (Kcnq1ot1, Igf2, and Peg3) and five that are maternally expressed (Igf2r, H19, Ascl2, Gtl2, and Kcnq1). The isolated RNA was used for reverse transcription to acquire cDNA (Table 1). A polymerase chain reaction (Table 2&3) was then used to amplify the DNA. The annealing temperature was dependent on the gene (Table 3). A DNA digest was then done using restriction endonucleases (Table 3) followed by gel electrophoresis on a 1% gel with 4µl of gel red and 1µl of 1000kb ladder at 90V for 180 minutes.

Step	Instructions	Product
Part A: DNAse Treatment	Treat 1ug of RNA with DNAse to eliminate genomic DNA concentration. • Combine 1μg of RNA and water to a total volume of 8 μl. • Combine 1μg of RNA and water to a total volume of 8 μl. • Add 1ul of DNAse buffer • Add 1ul of DNAse • Mix gently and keep at room temperature for 15 minutes then add 1μl of Stop Solution.	Sigma DNAse (AMP-D1)
Part B: Reverse Transcription I	Split the DNAse treated RNA into 4 groups: 3 positive and 1 negative reverse transcription • 1μl of 10 mM dNTP/sample • 1μl Random Hexamers/sample • 2.75 μl of the DNAse treated RNA • 8.25ul water • Incubate at 70°C for 10 minutes	Invitrogen,18427-013 Invitrogen, 48190011
Part C: Reverse Transcription II	Positive and Negative reverse transcription • 2μl 0.1M DTT per sample • 4μl 5x 1st Strand Superscript II Buffer • 1μl of Superscript II (Positive) • 1μl of water (Negative) Add 7 μl into each reverse transcription reactions	Invitrogen,18064-071
Part D: Program	Reverse Transcription Program • 42°C 50 Minutes • 45°C 10 Minutes • 50°C 10 Minutes • 55°C 10 Minutes • 70°C 5 Minutes • 4°C Hold	

Table 1. Reverse Transcription Protocol. Reverse transcription steps and catalog numbers.

Reagent	Amount	Product
Platinum Taq Polymerase	0.1µl	Invitrogen, 10966034
10X PCR Buffer, Minus Mg	2.5µl	Invitrogen, Y02028
50 mM MgCl2	0.75µl	Invitrogen, Y02016
10 mM dNTP	0.5µl	Promega, U1515
10µM Primers	0.5µl	
1:100 DNA	5µl	
Ultra-Pure Water	14.65µl	

Table 2. Polymerase Chain Reaction Protocol. PCR steps and catalog numbers.

Gene	Primer Sequence Forward (5'to 3')	Primer Sequence Reverse (5'to 3')	Annealing Temperature (°C)	New England Biolabs Restriction Endonuclease	New England Biolabs Catalog Number
H19	TGATGGAG AGGACAGA AGGGC	CTTGATTCA GAACGAGAC GGACT	60.0	Cac8I	R0579S
IGF2	ATCTGTGAC CTCCTCTTG AGCAG	GGGTTGTTT AGAGCCAAT CAA	55.0	MluCI	R0538S
Ascl2	TGAGCATCC CACCCCCCT A	CCAAACATC AGCGTCAGT ATAG	61.0	SfcI	R0561S
Kcnq1	CATCGGTGC CCGTCTGAA C	TGCTGGGTA GGAAGAGCT CAG	58.8	NlaIII	R0187S
Kcnq10t1	ATTGGGAA CTTGGGGTG GAA	GGCACACGG TATGAGAAA AGATT	56.5	StuI	R0187S
Peg3	AAGGCTCT GGTTGACA GTCGTG	TTCTCCTTGG TCTCACGGG C	60.0	HpyCH4III	R0618S
IGF2r	TGGAGACC TCACCCTCA TCTATTC	GCACACAGC AAGCATCTT CAG	60.0	TaqαI	R0149S
Gtl2	CCAAAGCC ATCATCTGG AATC	CAGCCCTGT GAGGTAGGA AC	53.4	SfcI	R0561S

Table 3. Annealing Temperatures.

Each fetus was sexed by using the DNeasy Blood and Tissue Kit previously mentioned. A PCR using Zfy and Xist primers (Table 4) with a 58°C annealing

temperature was run on the DNA that was extracted. The PCR product was then run on a 1% agarose gel using the same parameters previously. Xist was used for a positive control to detect if DNA was present and Zfy was used to determine the sex. If a band appeared on the gel for Zfy, this indicated the fetus was a male. If there was no band, this indicated a female.

Gene	**Primer Sequence Forward (5'to 3')**	**Primer Sequence Reverse (5'to 3')**	**Annealing Temperature (°C)**
Zfy	AAGATAAGCTTACAT AATCACATGGA	CCTATGAAATCCTTG CTGCACATGT	58.0
Xist	TTGCGGGATTCGCCT TGAT	TGAGCAGCCCTTAAA GCCAC	58.0

Table 4. Sex Determination. Primer sequences and annealing temperatures.

By using an unpaired t-test, growth parameters, parental weights, and fluid consumption were tested using GraphPad Prism software. The RNA sequence of single nucleotide polymorphisms and the ratio of males versus females in the two treatment groups were analyzed with a Fisher's Exact test on the GraphPad Prism software. The gene expression from the RNA sequencing data was analyzed with Cuffmerge and Cuffdiff, R statistical software, and Ingenuity Pathway Analysis.

To test if there was an interaction between independent variables and dependent variables an ANOVA analysis was performed. There was no interaction between fetal weight compared to the sire's weight ($p>0.05$) nor compared to the dam's weight

($p>0.05$). We did not observe an interaction between the dam's weight and litter size nor litter size and fetal weight ($p>0.05$).

CHAPTER III

RESULTS

3.1 Model of Male Alcohol Exposure

To examine the impact of prenatal alcohol exposure on parental patterns of inheritance, two strains of mice carrying distinct single nucleotide polymorphisms were utilized. This allowed us to track allelic patterns of DNA methylation and gene transcription. The B6(Cast7) strain of mice [121] possess portions of a *Mus musculus castaneus* (CAST) chromosome 7 and 12 (where at least 5 imprinting domains and more than 30 imprinted genes reside) bred onto a C57BL/6J (B6) background. When using F1 hybrid crosses between the B6(CAST7) strain and a C57BL/6J strain, we can distinguish maternal and paternal alleles using CAST and C57BL/6J polymorphisms that we have identified by either by primary sequence or database analysis. These polymorphisms allow the identification of parental alleles through either primary sequence analysis or restriction digest based assays (i.e. one parental allele is susceptible to digestion while the other is not). These strains of mice are both from a C57BL/6J strain background which is susceptible to alcohol-induced teratogenesis.

To investigate the impact of alcohol exposure on the male-inherited developmental program, an established mouse model of chronic, low-dose exposure was employed [120] Here, postnatal day 90, B6(Cast7) adult males were provided limited access to alcohol during a four hour window immediately after their sleep cycle [120]. Males were maintained on a 12-hour light/dark cycle (19:00-7:00) and provided access to either a solution of 10% (w⁄v) alcohol and 0.066% (w⁄v) saccharin (experimental) or

0.066% (w⁄v) saccharin alone (control) for four hours a day. During the 70-day treatment period, the average fluid consumption normalized to body weight was not significantly different between the two treatment groups (Figure 16, p= 0.0937).

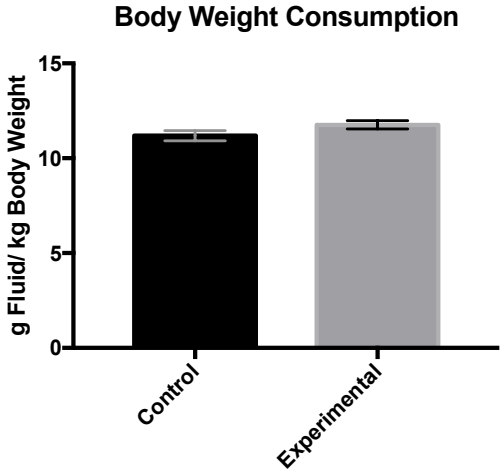


Figure 16. Fluid Consumption Relative to Body Weight. Water intake for the control mice versus ethanol solution intake normalized to body weight. There was no significant difference in two treatment groups. Unpaired t-test p=0.0937.

Further, over the experimental time course, an equivalent pattern of consumption was observed between both treatment groups (Figure 17, experimental p=0.2079 control p=0.3888). Based on previous reports, the expected blood ethanol content (BEC) are estimated to be 200 mg/dL [120]. For reference, the estimated BEC exceeds the legal limit in the U.S. of 80 mg/dL while driving. A BEC of 150 mg/dL can induce symptoms including a severe loss of muscle control, vomiting, and a major loss of balance in humans [123].

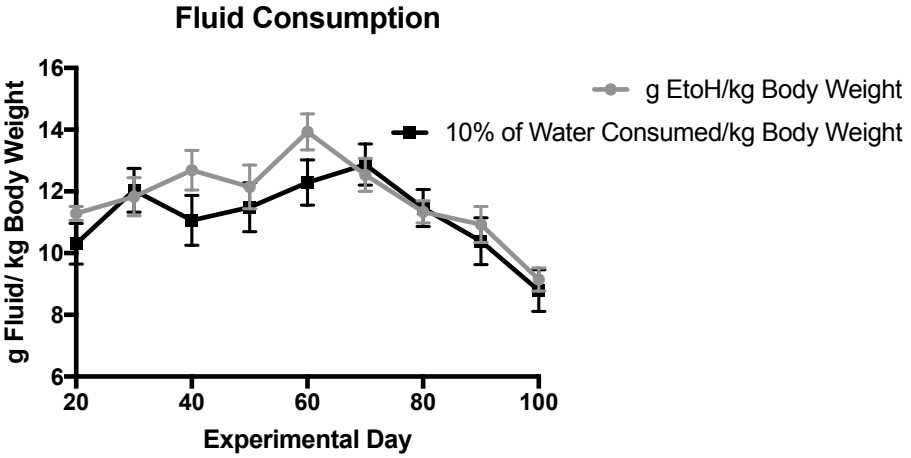


Figure 17. Fluid Consumption per Kilogram Bodyweight Over Time. The grams of ethanol and 10% of water consumed over an 80-day period with nine time points. Linear Regression was used for statistical analysis.

Given the known association between excessive weight gain and altered epigenetic programming in sperm [90] we first wanted to examine if alcohol consumption was associated with changes in body mass. No significant differences in the weight or body condition of the experimental males were observed for the duration of treatment (Figure 18, p=0.5156).

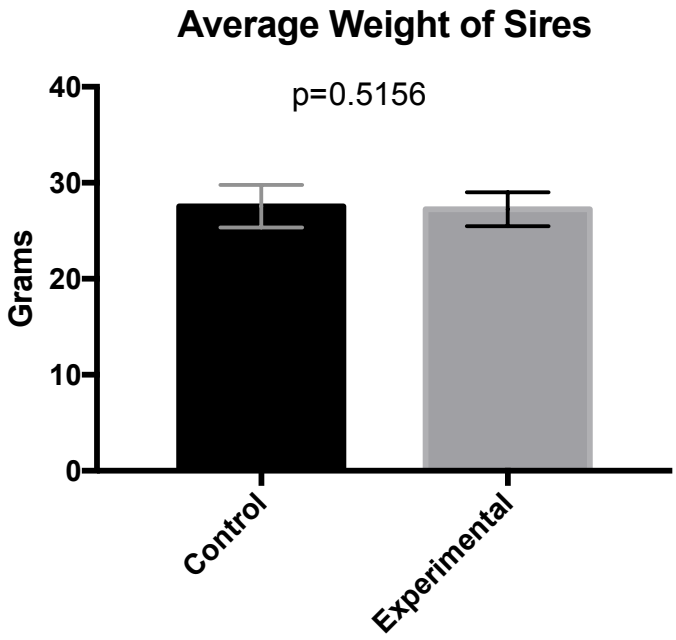


Figure 18. Sire Weight. There was no significant difference between the sires' weight (experimental sire n=10 control sire n=8). An unpaired t test was used for the statistical analysis.

Once consistent patterns of drinking had been established, males were maintained on this protocol for a period of 70 days, which corresponds to the length of approximately two complete spermatogenic cycles, and ensures that both pre-meiotic and postmeiotic sperm were exposed to alcohol [122,124]. To clear mature, pre-existing sperm formed prior to Alcohol exposure, males were bred to non-experimental dams 35 days after consistent drinking had been established. Once the 70-day milestone had been surpassed, males were mated to unexposed dams. No differences in the weights of experimental or control dams were observed (Figure 19, p=0.5361).

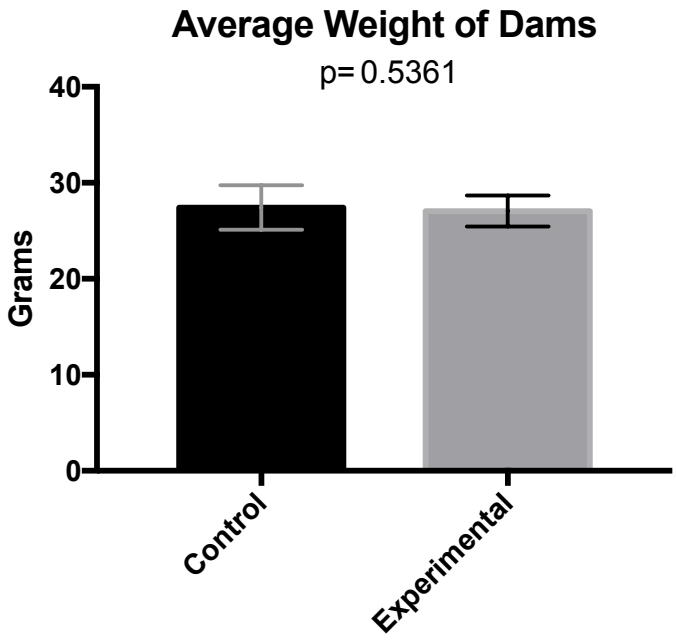


Figure 19. Dam Weight. There was no significant difference between the dams' weight (experimental dam n= 22 control dam n=16). An unpaired t test was used for the statistical analysis

3.2 Alterations in Fetal Growth Parameters Arising as a Consequence of Preconception Male Alcohol Exposure

After confirmed matings (presence of a vaginal plug), dams were separated, and fetuses collected at day 14.5 of gestation. The GD14.5-time point was selected due to our technical experience reliably excising the fetal component of the placenta away from maternal tissues, in order to avoid contaminating maternal cells and accurately assess placental patterns of gene expression [125]. During this dissection, the uterus was removed, and the maternal decidua separated from the fetal interface. Subsequently, the length of the fetus and weight of the gestational sac, placenta and fetus were recorded from 38 (control n=16, ethanol n = 22) litters with 260 pups examined (control n=113,

ethanol n=147). From this data, chronic male alcohol exposure was determined to be associated with a significant 4% reduction in the weight of the gestational sac (Figure 20a, p=0.0159) and a 5% reduction in fetal weight (Figure 20b, p = 0.0217). These observations are similar to a previous study, which also identified reduced weight at postnatal days 35-42 in the offspring of alcohol-exposed males [126]. Further, this growth restriction is similar in magnitude to the one previously reported by Gundogan et al., examining the offspring of ethanol-exposed dams [127]. Fetal crown-rump length decreased by 3% (Figure 20c p=0.0015). There was no significant difference between the placental weight between the two groups (Figure 20d, p=0.2981).

Fetal / placental weight ratios have often been considered as a crude proxy to measure placental efficiency [128,129]. When we derived a ratio of fetal weight relative to placental weight, an 8% decrease in the relative size of the placenta in the offspring sired by alcohol-exposed males was observed (Figure 20e, p=0.0101). These results are consistent with those reported in animal studies examining assisted reproductive technologies [130]; while diametrically opposite to the observed impacts of maternal diabetes / obesity on fetal-placental growth [129,131].

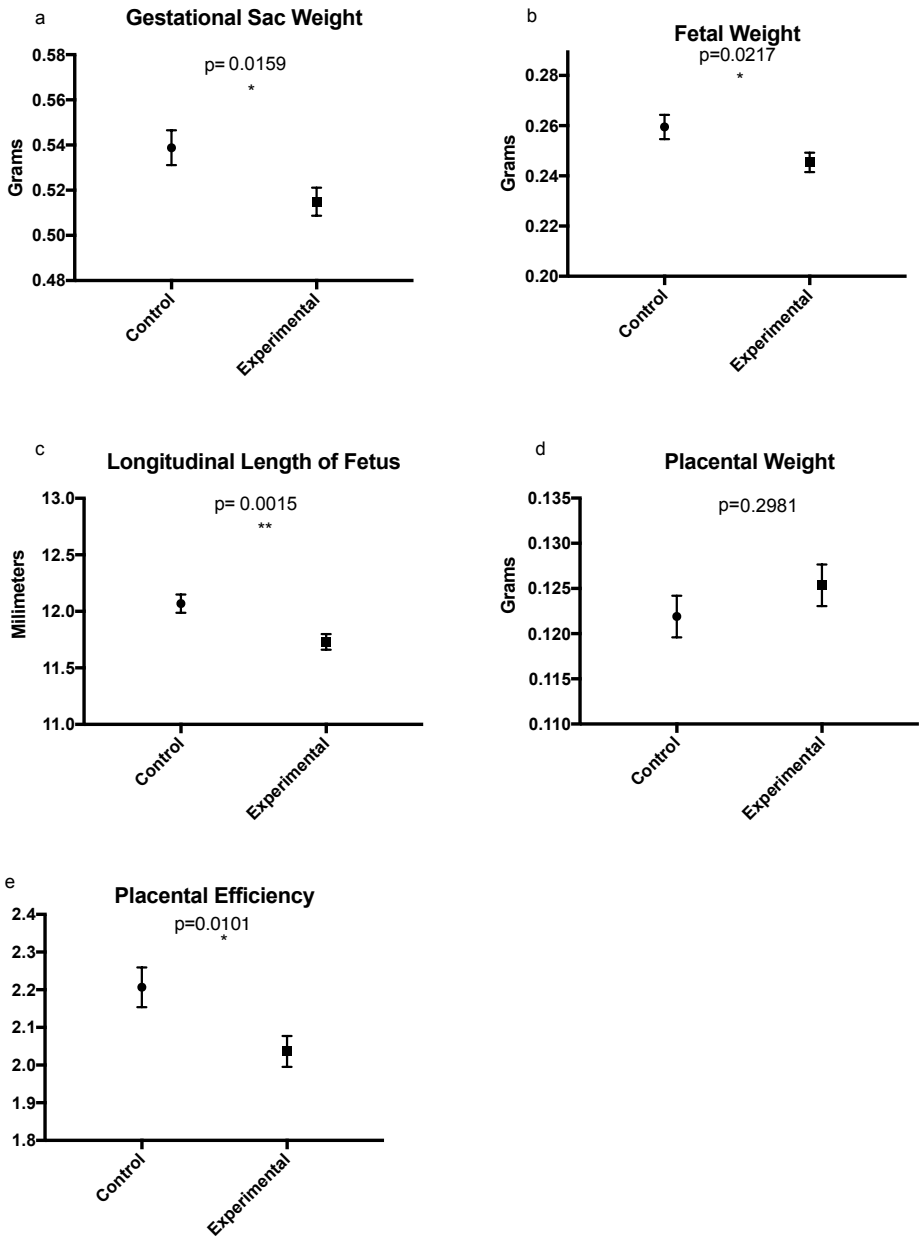


Figure 20. Fetal Parameters. Gestational sac weight (b, control n=97, ethanol n=106), fetal (a, control n=112, ethanol n=135) and longitudinal length (c, control n=110, ethanol n=144) of the fetus were all significantly different from the control. There was no significant difference of the placental weight between the two treatment groups (d, control n=114, ethanol n=147); however, there is a significant difference in placental efficiency (e, control n=110, ethanol n=135). An unpaired t-test was used for statistical analysis.

The sex of the fetus may also be an important factor influencing the observed differences. Therefore, we stratified our data based on fetal sex, which was determined using PCR. We first examined our dataset to determine if preconception alcohol exposure was associated with a difference in fetal sex. No significant difference in fetal sex was observed between the treatment groups (Figure 21, p=0.3756).

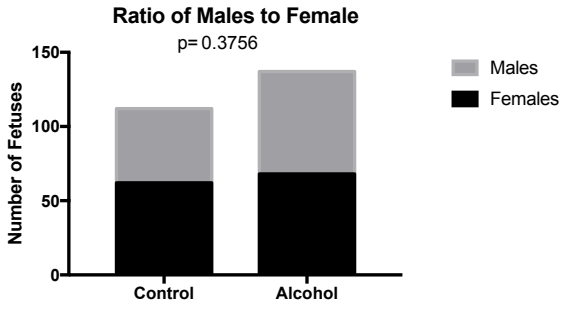


Figure 21. Ratio of Sexes. There was no significant difference between the number of females and males in each treatment group. (control females n=62 control males n=50, ethanol females n=68 ethanol males n=67) Fisher's Exact test was used for statistical analysis.

We next sought to determine if fetal sex was a significant modifier of the observed alterations in fetal growth. There was a sex-specific difference in the weight of the gestational sac observed within the experimental treatment group with a 7% decrease in gestational sac in female offspring (Figure 22b, p=0.0095). However, when comparisons were made between treatment groups, the gestational sac of male offspring was not significant from sires in the alcohol treatment group compared to the offspring of sires receiving control treatments. Although, the gestational sac of female offspring sired by alcohol-exposed males was decreased by 6% compared to the female offspring of control males (Figure 22d, p =0.0068).

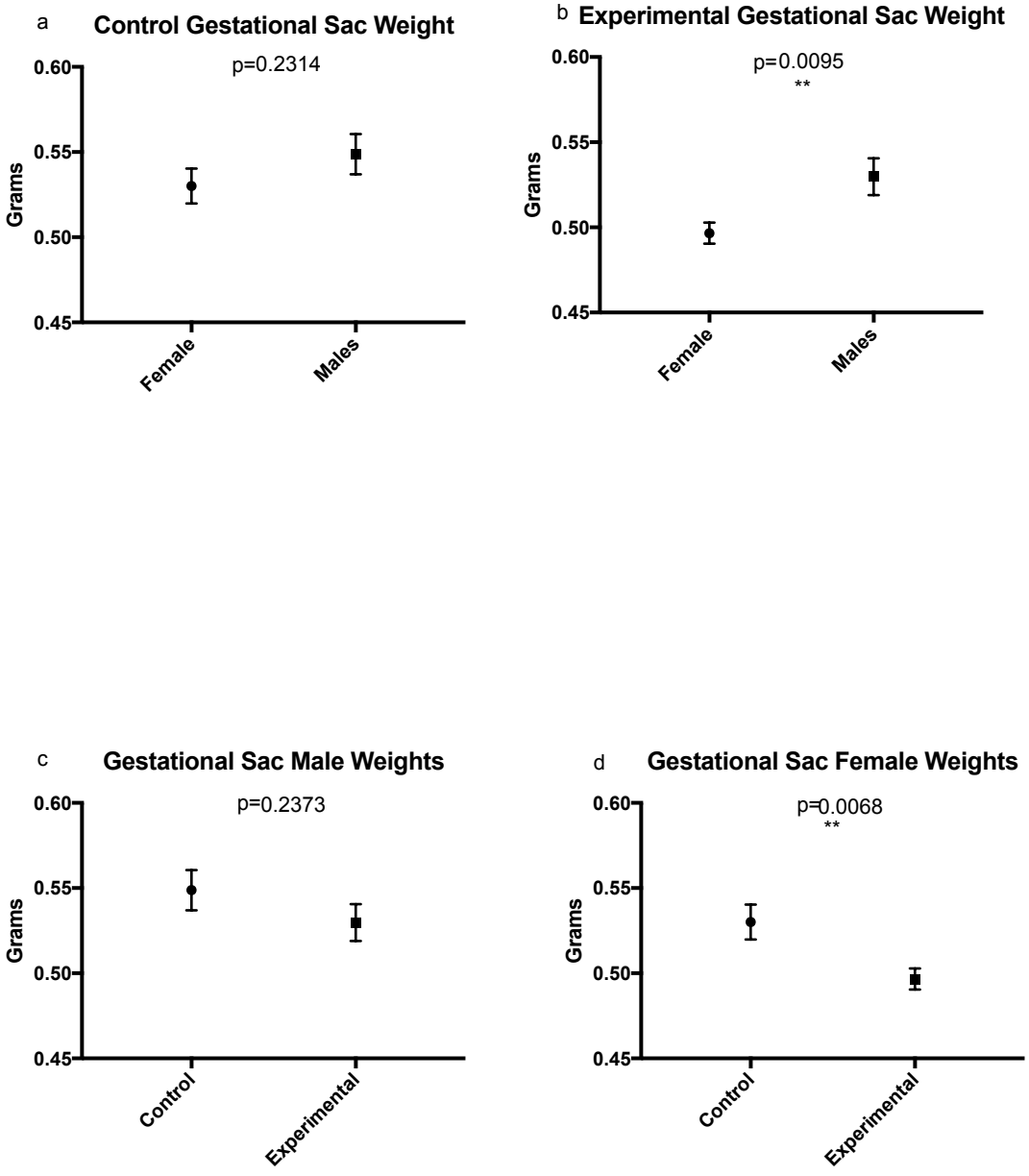


Figure 22. Gestational Sac Weight. The weight of the gestational sac was significantly different within the sexes of the experimental treatment groups, but not within the control group. However, when comparisons were made between treatment groups, a significant reduction in gestational sac weight was observed for female offspring, but not for male offspring. (Experimental gestational sac weight: female n=50, male n=51, control gestational sac weight: female n=50 male n=44). An unpaired t test was used for the statistical analysis.

When comparisons were made between the fetal weights of male and female offspring, we observed dramatic sex specific effects in the offspring of alcohol-exposed males. For example, no significant differences in fetal weights were observed between male and female offspring within the control treatment; however female fetal weight was 10% lower than male weight in the offspring of the alcohol treatment group (Figure 23a-b, p=0.2671, p=0.0020 respectively). These results indicate that female offspring were more profoundly impacted by preconception alcohol exposure than male offspring.

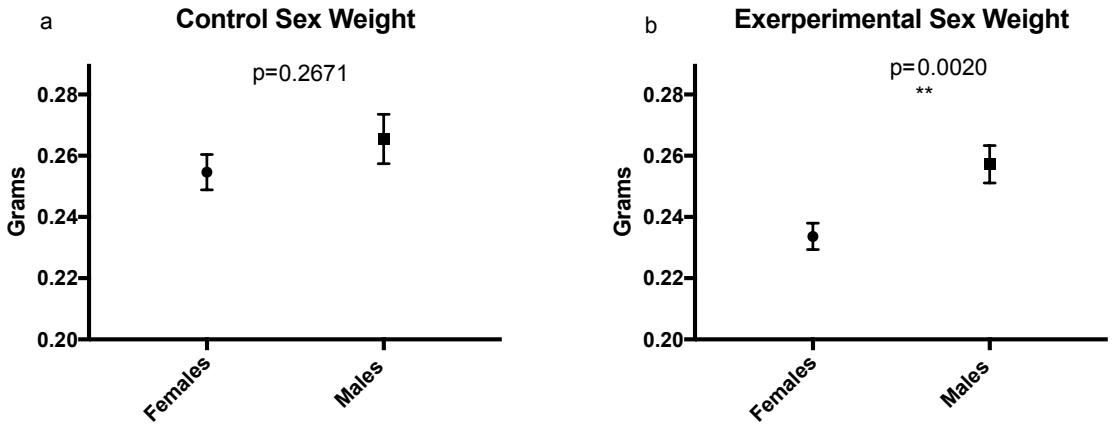


Figure 23. Fetal Weight Within Treatment Groups. The experimental treatment had a significant effect on female offspring in comparison to the male offspring while there was no significant difference in fetal weight in the control treatment. (Experimental fetal weight: female weight n=65 male weight n=67, control fetal weight: female weight n=62 male weight n=50). An unpaired t test was used for the statistical analysis.

Indeed, when sex-specific comparisons were made between experimental treatments, this is what we observed. Whereas no differences in fetal weights emerged amongst male offspring, females derived from alcohol-exposed males exhibited an 8%

decrease in fetal weight relative to those sired by control males (Figure 24a-b, p=0.4096, p=0.0039).

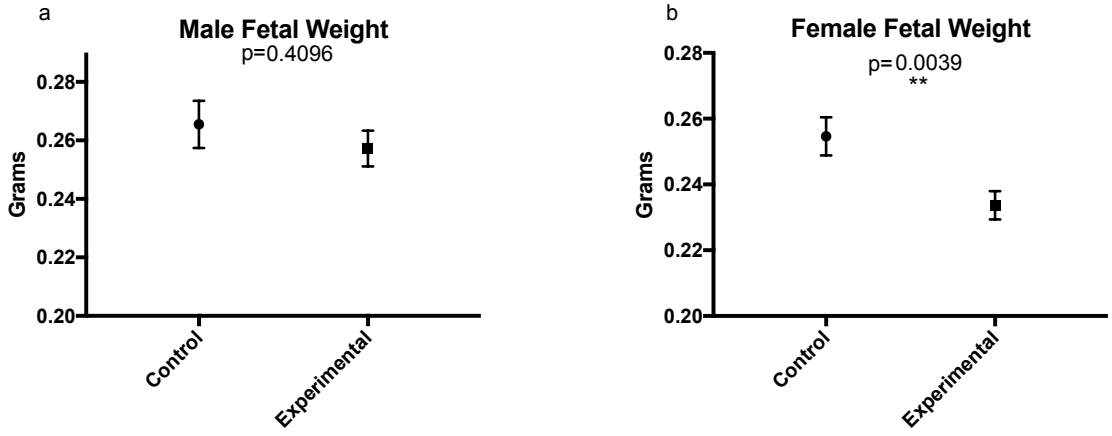


Figure 24. Fetal Weight. There was no significance in the weight in male offspring between the alcohol and control treatment groups; however, there was a significant decrease in female weight in the offspring sired by ethanol exposed males. (Experimental fetal: female weight n=68 male weight n=67, control fetal: female weight n=62 male weight n=50). An unpaired t test was used for the statistical analysis.

There was a sex-specific differences in crown rump-length within the experimental group, but not within the control group or between the sexes of the treatment groups (Figure 25a-b, p=0.9053, p=0.0133 respectively). The females sired from experimental males had a 3% significant decrease in comparison to males sired from experimental males (Figure 25b, p=0.0133).

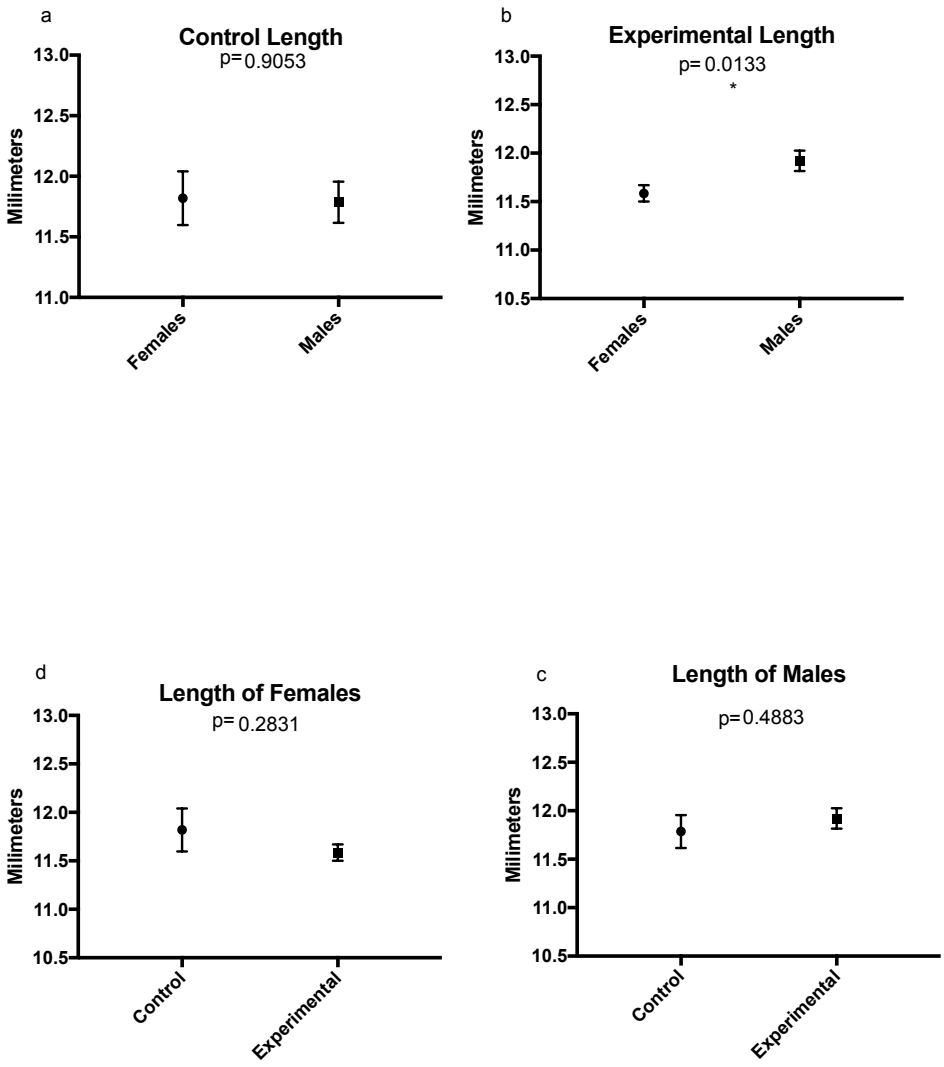


Figure 25. Longitudinal Fetal Length. There was a significant difference in fetal length within the treatment group, but not within the control treatment group. Nor was there a significant different with the sexes between the treatments groups. (Experimental fetal length: female n=77 male n=73, control fetal length: female n=60 male n=61). An unpaired t test was used for the statistical analysis.

There was no significant difference in the placenta weight between or within each treatment group (Figure 26, $p>0.05$).

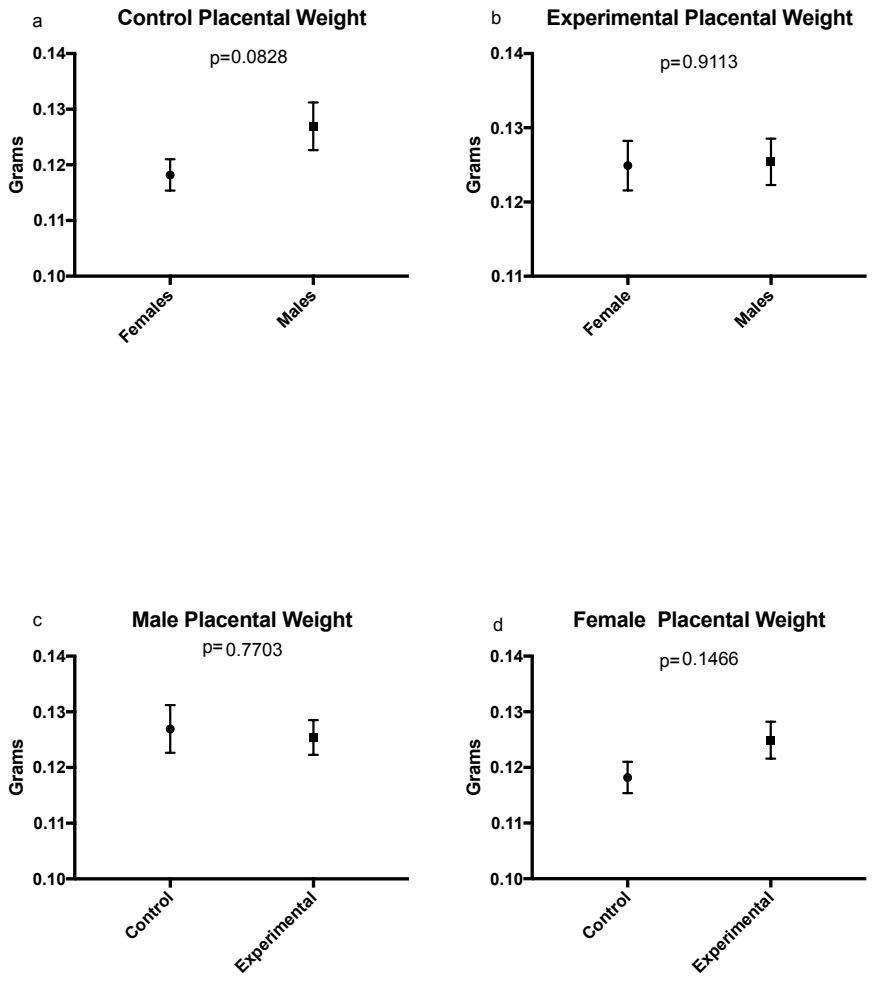


Figure 26: Placental Weight: There was no significant difference in the experimental or control sex weights nor in the sex between the two treatment groups. (Experimental: female n=79, male n=57, control: female n=73 male n=48). An unpaired t test was used for the statistical analysis.

When we examined placental efficiency for sex-specific effects, we did observe a significant difference (Figure 27b, p=0.0038). The placental efficiency of the female offspring of alcohol exposed sires were significantly 12% smaller than either the male offspring within the alcohol treatment group and compared to females sired by control males with a 14% decrease. (Figure 27a, d, p=0.0038, p=0.0001).

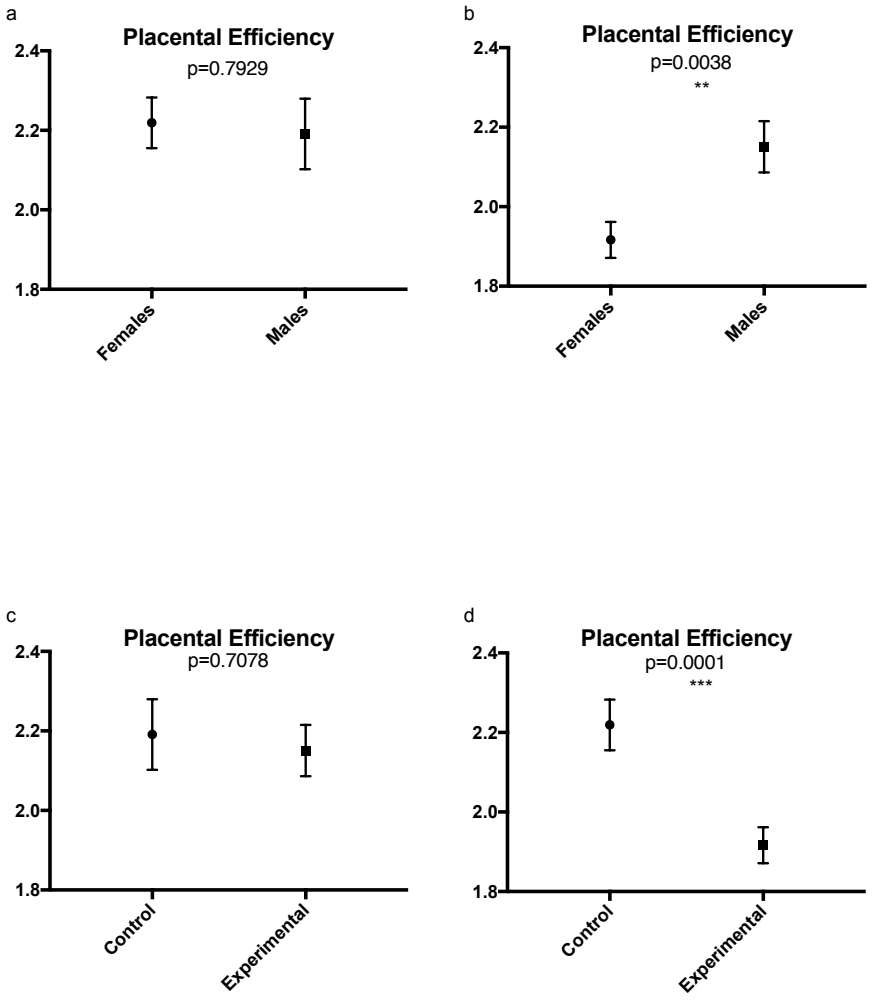


Figure 27. Placental Efficiency. There was no significant difference in control placental efficiency nor the male offspring compared to the two treatment groups. However, there was a significant difference within the experimental group and between the female offspring between the two treatment groups. (Experimental: female n=66, male n=69, control ratio: female n=61 male n=49). An unpaired t test was used for the statistical analysis.

3.3 Altered Epigenetic Inheritance in a Model of Preconception Male Alcohol Exposure

To establish if chronic alcohol exposure could influence the male-inherited epigenetic program, and be linked to the observed growth restriction, we next examined the DNA methylation profiles of paternal sperm. After a minimum of three matings, breeder males were sacrificed, and sperm collected using a modified swim-up procedure [132] No significant difference in sperm concentration was observed between the treatment groups (Figure 28a, p=0.2069). There was also no significant difference in the quantity of living sperm between treatments (Figure 27b, 0.6261).

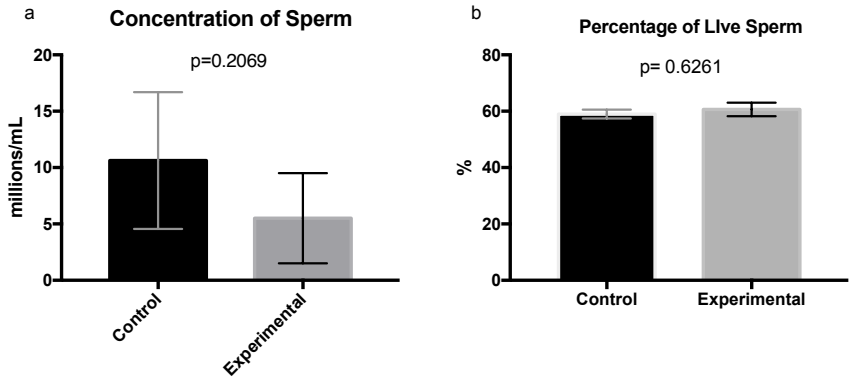


Figure 28. Sperm Analysis. Concentration of Sperm and percentage of live sperm compared to dead sperm. (Experimental n=4, control group n=3). Unpaired t-test was used for statistical analysis.

After isolation of sperm, the DNA methylation profiles of three control and three experimental males were independently examined using bisulphite mutagenesis and second-generation deep sequencing [133,134]. The Methyl-Sequencing technique we employed queried the methylation status of 7-8 million unique CpG sites,

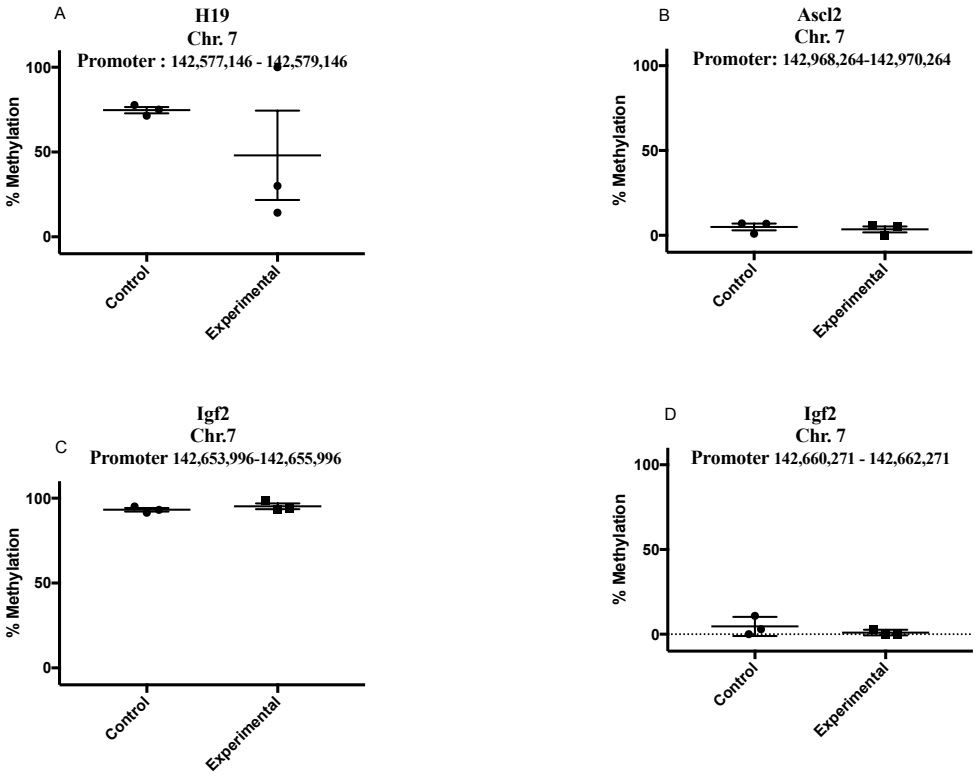


Figure 29. Percentage of Methylation at Promoter Regions. The percentage of methylation in the promoter regions H19, Ascl, and Igf2 was not statistically significant. Unpaired t-test was used for statistical analysis.

which covers virtually all CpG islands, gene promoters, genetic regulatory elements, gene bodies, and repetitive DNA sequences within the mouse genome.

For statistical comparison, the methylation data was broken down into promoter region, base pair, and tiling (1000bp) comparisons. In comparisons of the promoter regions, no differences in DNA methylation were observed for H19, Ascl2, or Igf2 (Figure 29, $p>0.05$).

Using base pair comparisons, no differences were observed within the regulatory regions of Peg3, or 15 locations within the regulatory region of Igf2r (Figure 29, $p>0.05$).

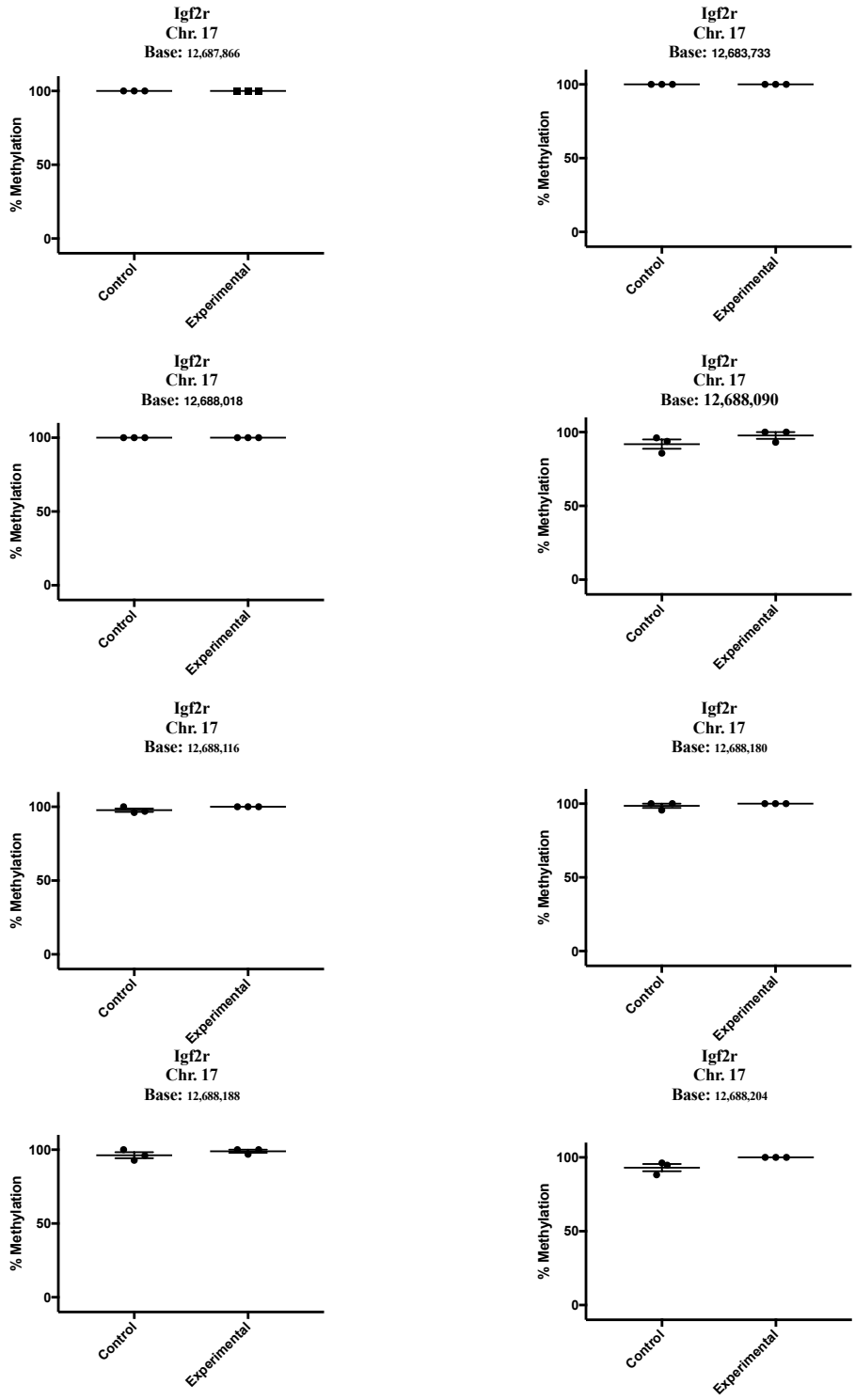


Figure 30. Percentage of Methylation at Base Pairs. The 15 different base pairs that were identified in Igf2r and one base pair from Peg3 had no significant percentage in methylation. Unpaired t-test $p>0.05$.

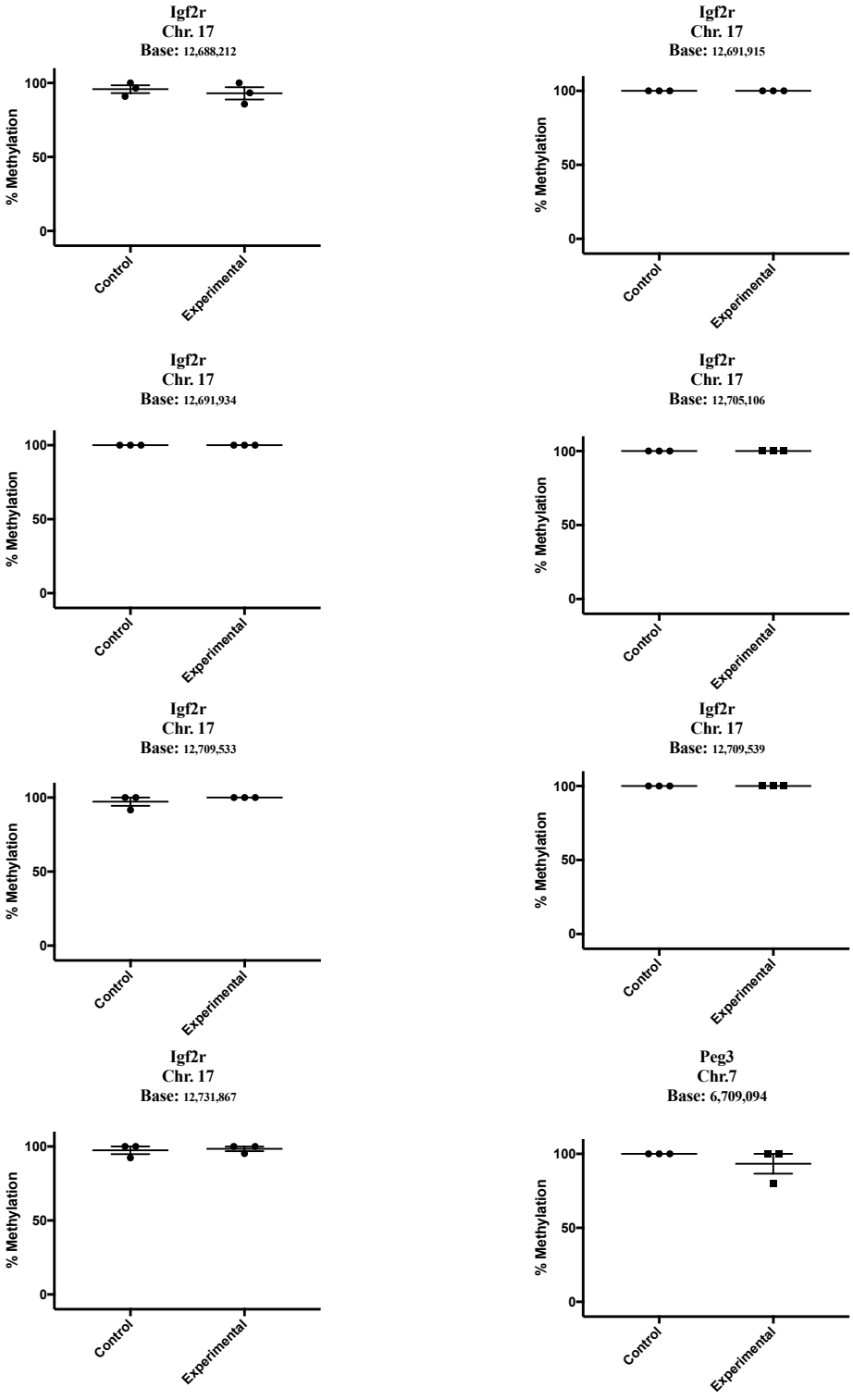


Figure 30. Continued.

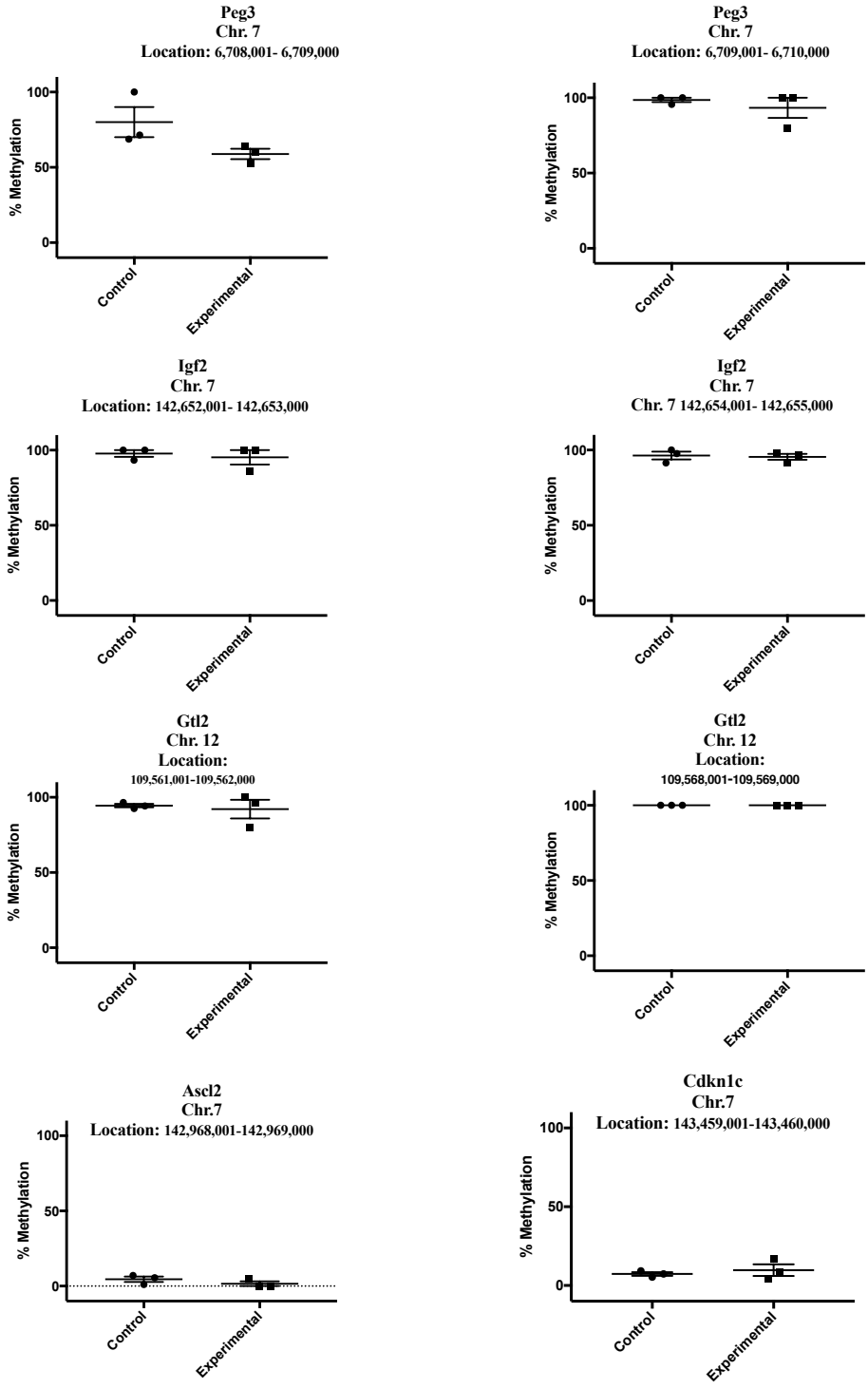


Figure 31. Percentage of Methylation Tiling (1000bp). None of the candidate genes were significantly different in the percentage of methylation. Unpaired t-test $p>0.05$.

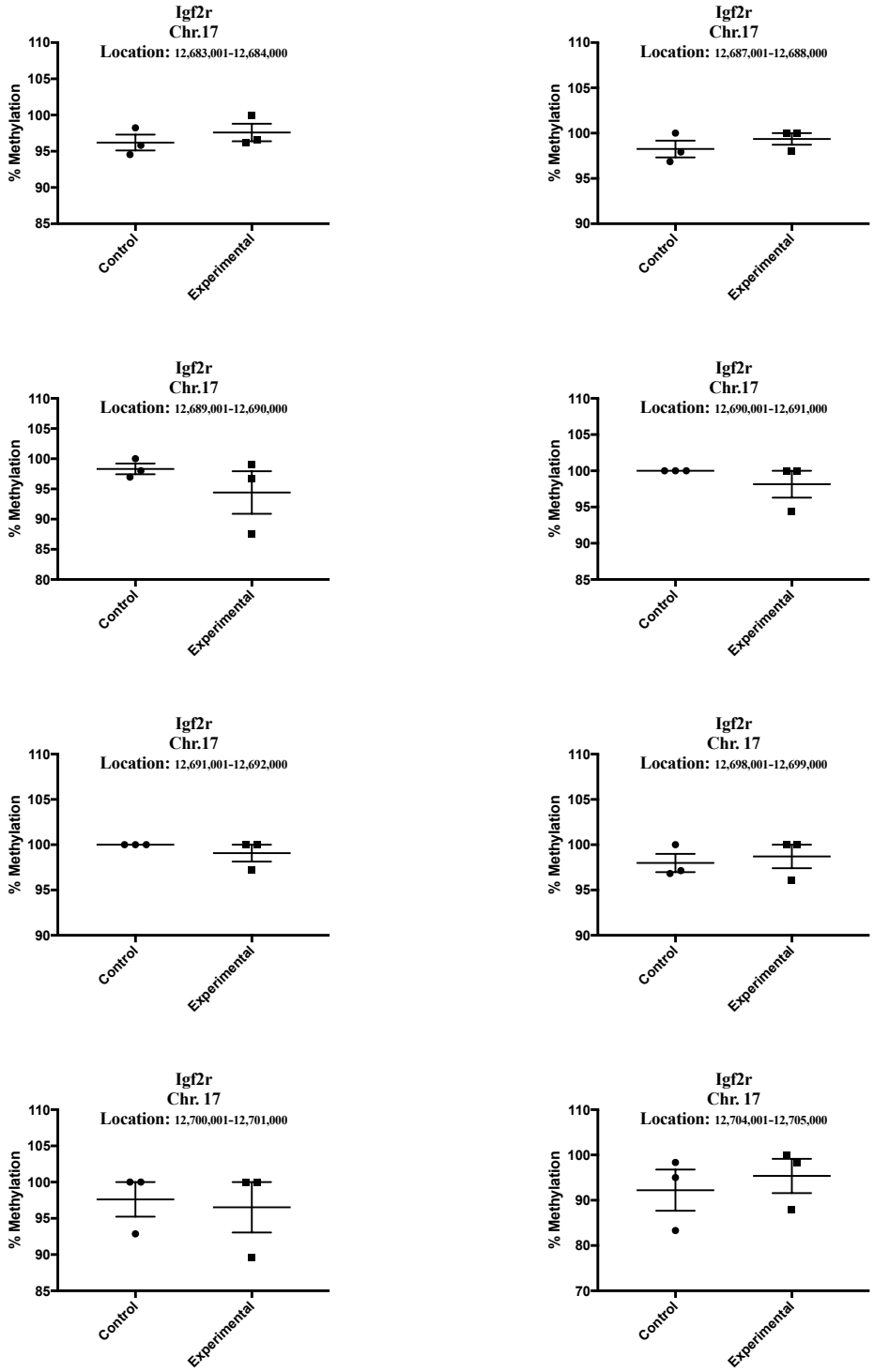


Figure 31. Continued.

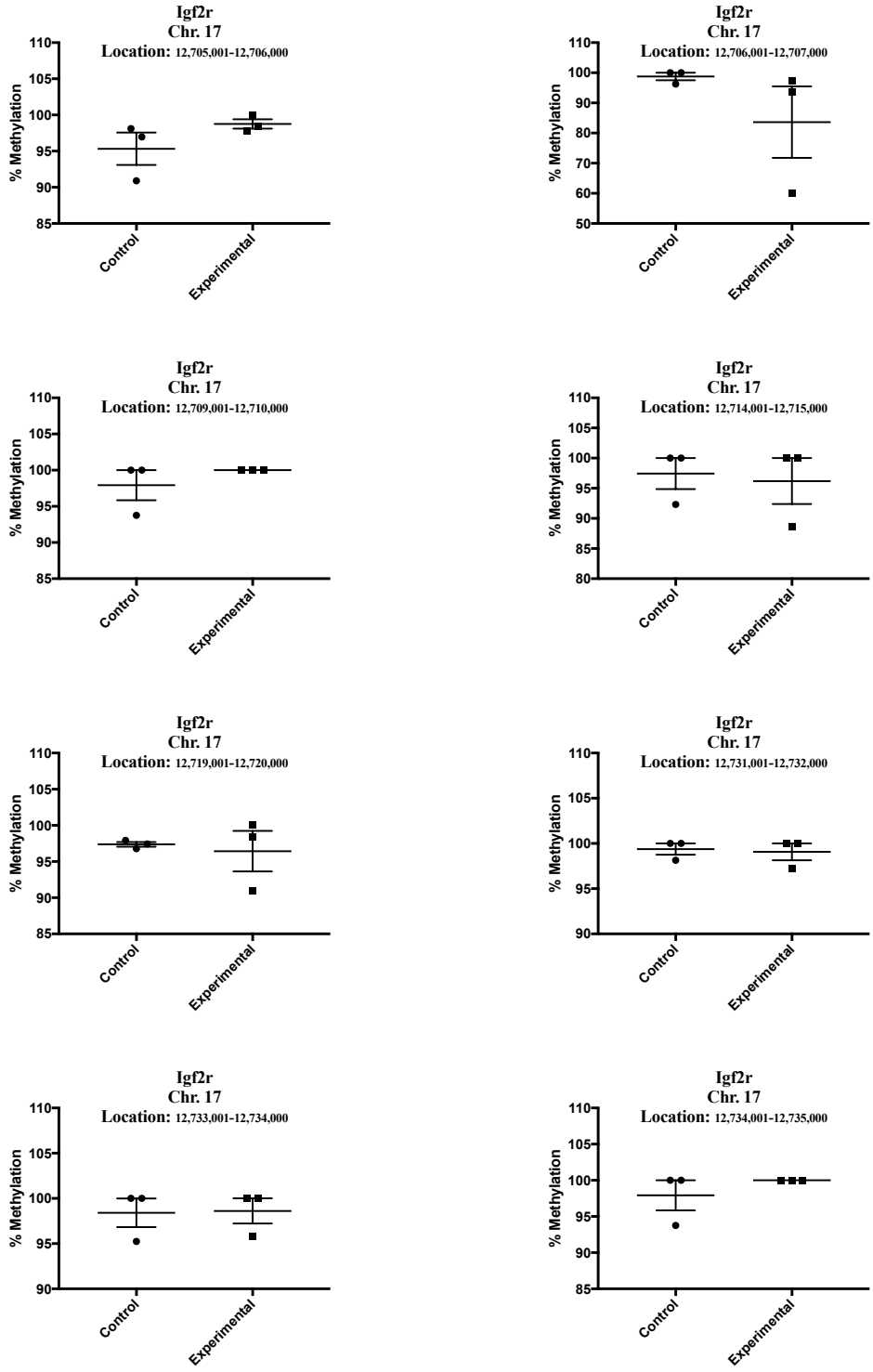


Figure 31. Continued.

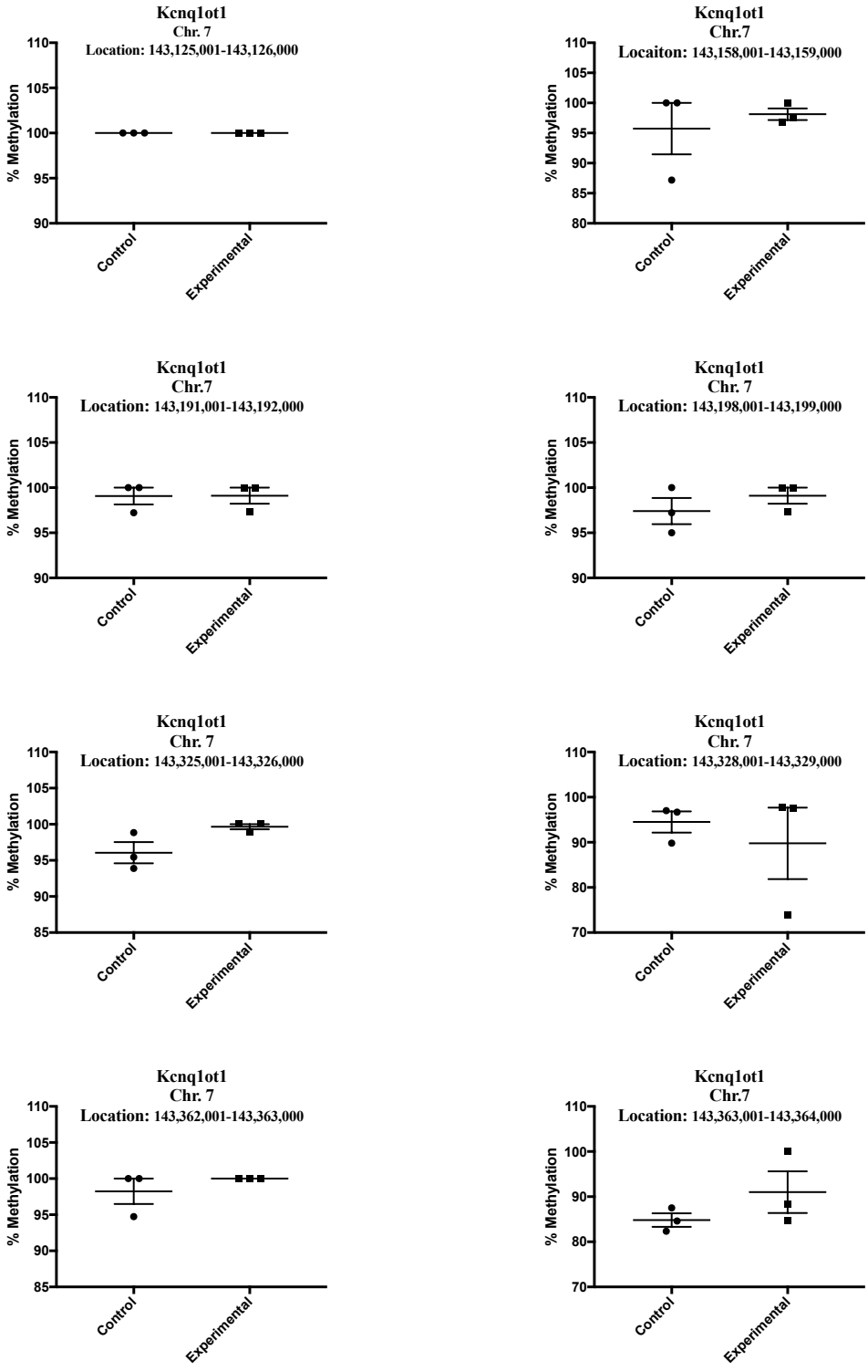


Figure 31. Continued.

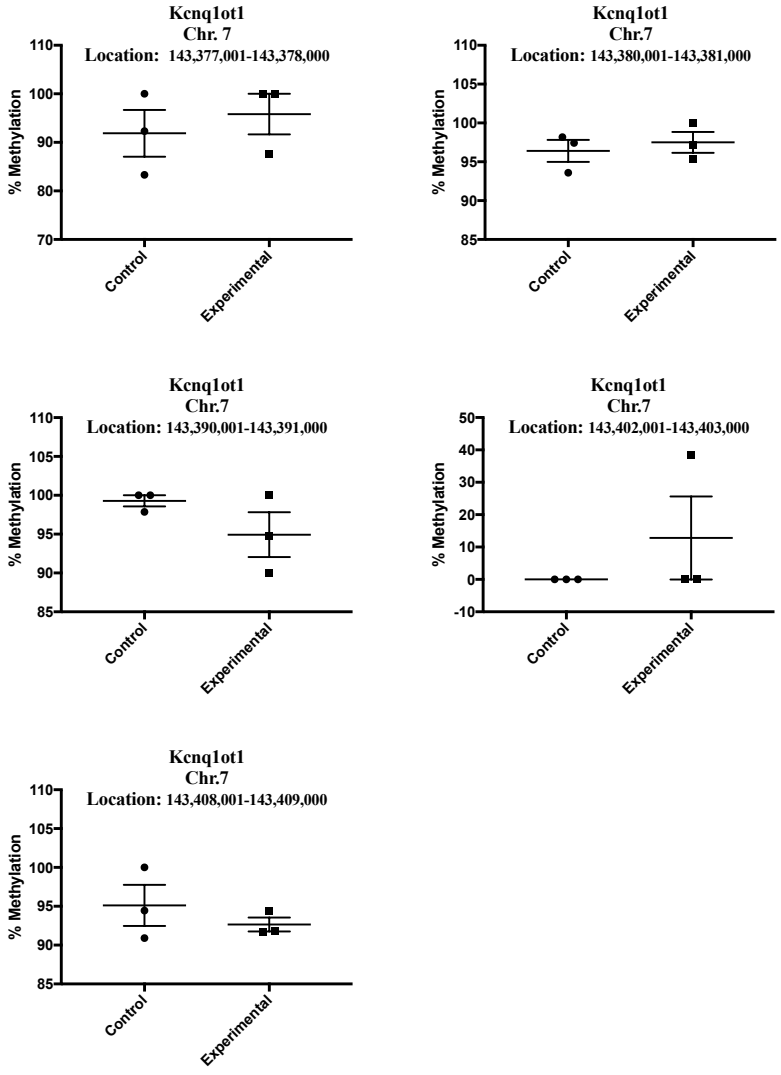


Figure 31. Continued.

Using tiling comparisons where the genome is broken into 1000 bp segments, and statistical comparisons made between regions, none of the candidate genes exhibited any statistically significant changes (Figure 31, $p>0.05$).

We next sought to determine if we could detect any alterations in the expression of our candidate-imprinted genes. The fetal placenta plays a critical role in controlling maternal-fetal resource allocation and mediating fetal growth [135,136]. Given the observed alterations in growth parameters of the placenta, we began our examination with this tissue. We first isolated RNA from placental samples and conducted deep sequencing analyses of the individual transcriptomes. Four samples (two males and two females) were analyzed from each of the control and experimental treatment groups. Total RNA was purified using an RNeasy Mini kit (Qiagen), and samples sequenced on an Illumina HISEQ 2500 (paired-end 100 bp). RNA expression levels were analyzed with Cuffmerge and Cuffdiff, statistical analysis performed with R, and data analyzed using Ingenuity Pathway Analysis.

From this dataset, we did not detect any significant changes in the expression of our candidate-imprinted genes (Figure 31, $p>0.05$). When we mined the sequencing data set for alterations in the representation of single nucleotide polymorphisms, we observed alterations in the abundance of the paternal allele of H19, which exhibited a significant decrease in transcripts derived from the paternal allele (Figure 32g, $p<0.0001$). In contrast, Cdkn1c exhibited a significant increase in the contribution of the normally silent paternal allele (Figure 32h, $p=0.0436$). We were able to follow two SNPs in the Peg3 and Igf2 genes, and one SNP in Gtl2 and Ascl2 genes (Figure 32, $p>0.05$)(Table 5). However, none of these demonstrated any significant differences.

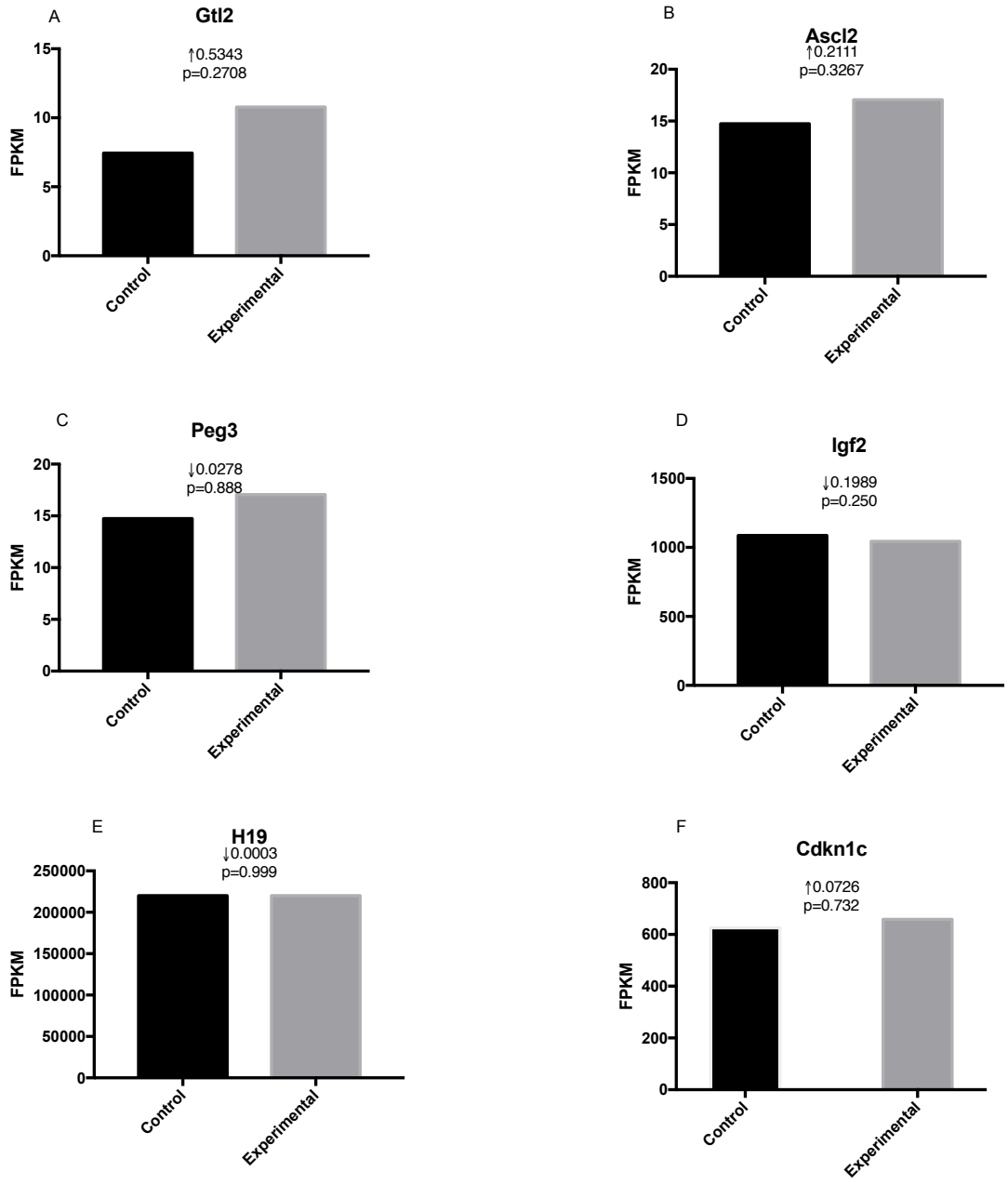


Figure 32: Gene Expression of Candidate Genes: There were no significant differences in the expression of expression of our candidate-imprinted genes within the placental samples examined. Unpaired t-test $p>0.05$.

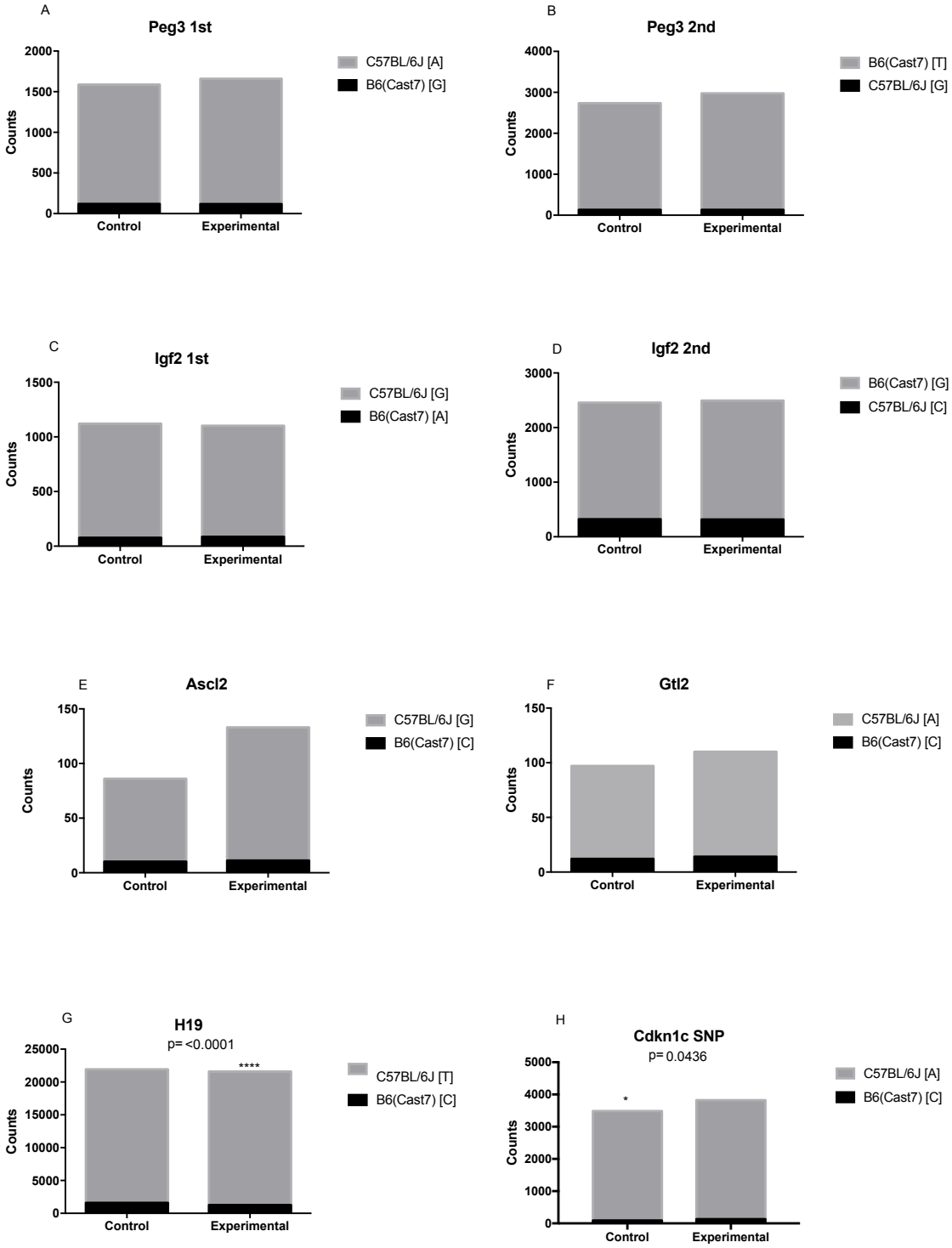


Figure 33: Analysis of Single Nucleotide Polymorphisms. SNPs within the transcripts of our candidate imprinted genes. The only significant alterations in observed SNPs were in H19(g, $p<0.0001$) and Cdkn1c (h, $p=0.0426$). Unpaired t-test.

Gene	B6Cast7			C57BL/6J		
	Base	Number		Base	Number	
		Control	Experiment		Control	Experiment
Peg3 (1)	G	119	117	A	1469	1542
Peg3 (2)	T	2604	2844	G	131	130
Igf2 (1)	A	77	86	G	1043	1016
Igf2 (2)	G	2135	2178	C	320	316
Ascl2	C	10	11	G	76	122
Gtl2	C	12	14	A	85	96
H19	C	1603	1262	T	20324	20342
Cdkn1c	C	87	126	A	3397	3696

Table 5. SNPs. List of all the SNPs examined. Only H19 ($p<0.000$) and Cdkn1c ($p=0.0436$) were found to be significantly different. Fishers Exact Test.

Previous studies employing an in utero model of prenatal alcohol exposure in rats have reported disruptions in the expression of the imprinted gene Dio3 [137]. In this study, they reported parent of origin differences in the expression of Dio3 within the frontal cortex and hippocampus. When we examined these same genes in our dataset, we observed an increase in expression in two imprinted genes from this cluster; Dio3 and Mirg (Figure 33, $p=0.0017$ and $p=0.0277$ respectively). Unfortunately, no SNPs could be identified to confirm parental contributions. Interestingly, alterations in the expression of these genes were dependent upon the sex of the fetus. Expression of Dio3 was significantly increased in the female offspring of alcohol exposed males, whereas the

male offspring were not different between experimental treatments (Figure 34B, p=0.001).

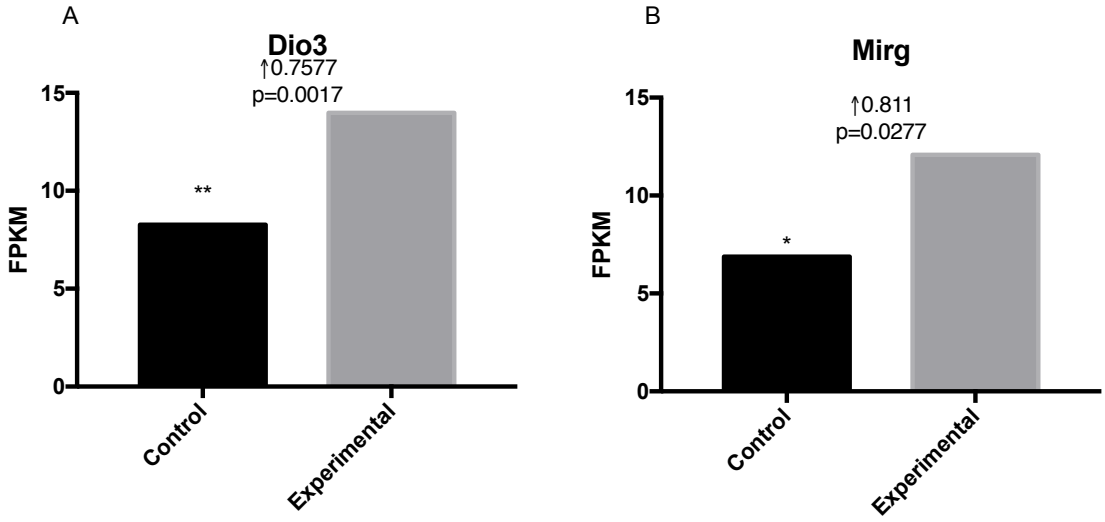


Figure 34. Gene Expression of Dio3 and Mirg. Dio3 and Mirg exhibited a significant increase within the placentas of offspring sired by alcohol-exposed males. Analyses were conducted using the statistical program Cuffdiff.

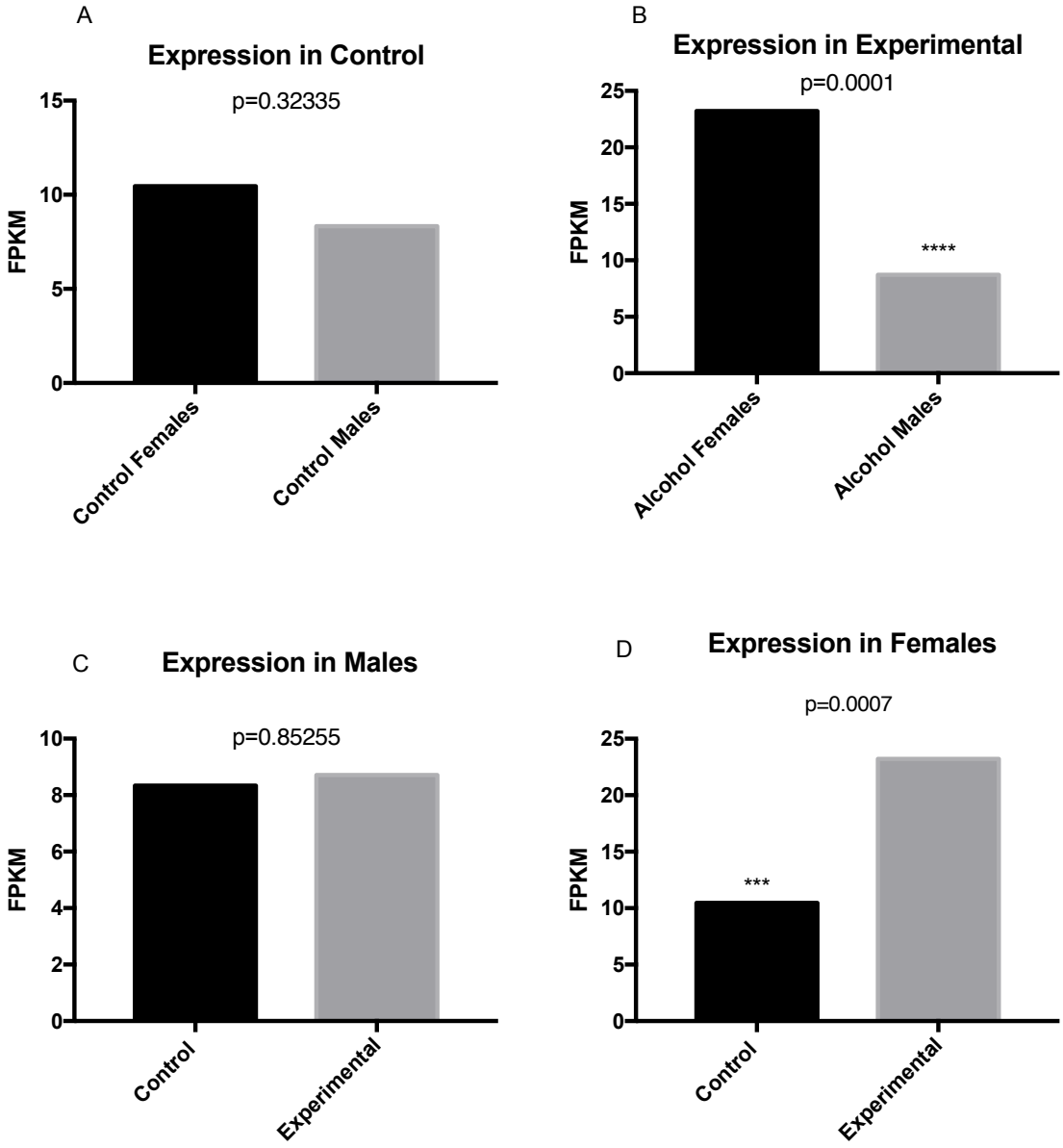


Figure 35. Sex-Specific Alterations in Gene Expression. The female offspring sired from experimental fathers exhibited a significant increase in the expression of Dio3, relative to male offspring. This increase in expression was also higher than those observed within the female offspring of control males. Analyses were conducted using the statistical program Cuffdiff.

Of the candidate imprinted genes examined, we have developed restriction digest-based assays to examine parental patterns of gene expression (Figure 36). For Gtl2, Peg3, H19, Igf2, Igf2r, Kcnq1, Kcnq1ot1, and Ascl2 we expanded our analyses to test 14 random placental samples with equal representation of males and females. The maternally imprinted genes are: Kcnq1ot1, Igf2, and Peg3, whereas paternally imprinted genes are: Igf2r, H19, Kcnq1, Ascl2, and Gtl2. The only gene to exhibit biallelic expression was Kcnq1ot1 (Figure 356), which exhibited increased maternal expression in three placental samples derived from alcohol-exposed males (A352, A341L4, and A341R5). The remaining seven candidate genes did not exhibit any detectable alterations in the regulation of imprinted alleles.

a

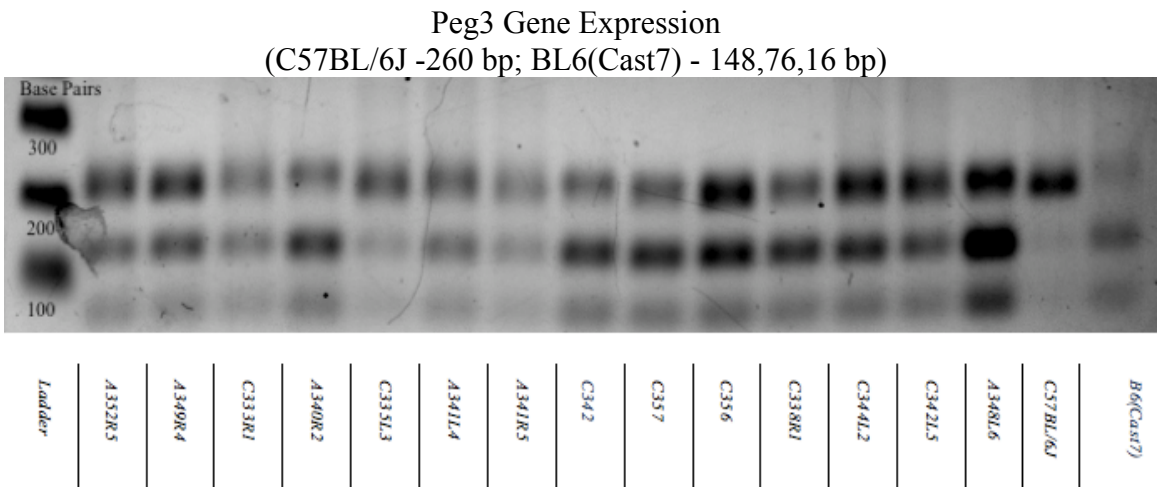
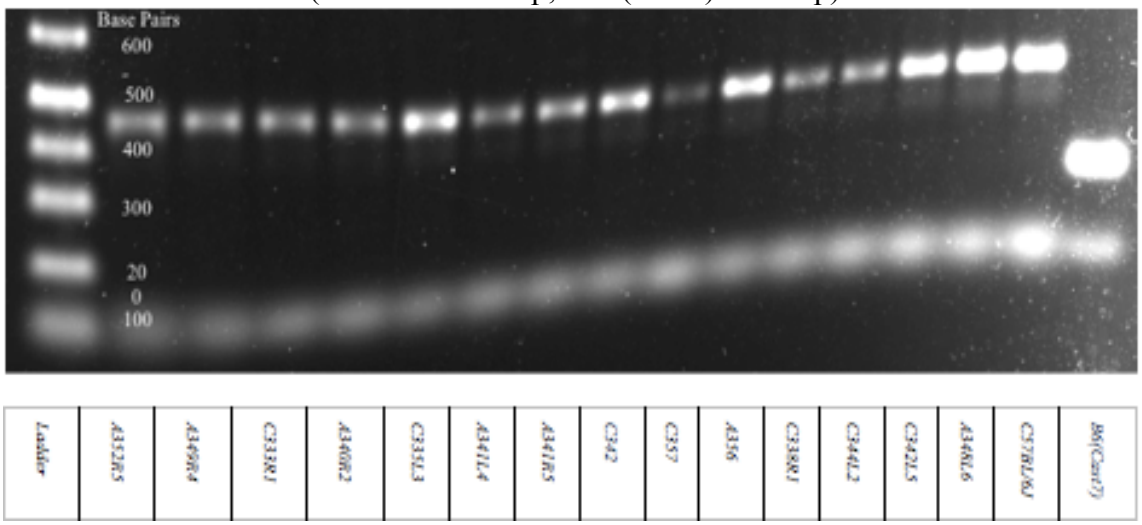


Figure 36. Restriction Digest Gene Expression. The samples in the above gene expression images are in the following order: A352R5, A349R4, C333R1, A340R2, C335L3, A341L4, A341R5, C342, C357, A356, C338R1, C344L2, C342L5, A348L6, C57BL/6J, L6(Cast7). The red circles (h) show some expression from the maternal allele in Kcnq1ot1. (A-alcohol male offspring, C- control male offspring, R- right uterine horn, L- left uterine horn, number indicates what dam they were from)

b

Ascl2 Gene Expression
(C57BL/6J 474 bp; BL6(Cast7) - 266 bp)



c

Kcnq1 Gene Expression
(C57BL/6J - 97,76 bp; BL6(Cast7) - 113,76 bp)

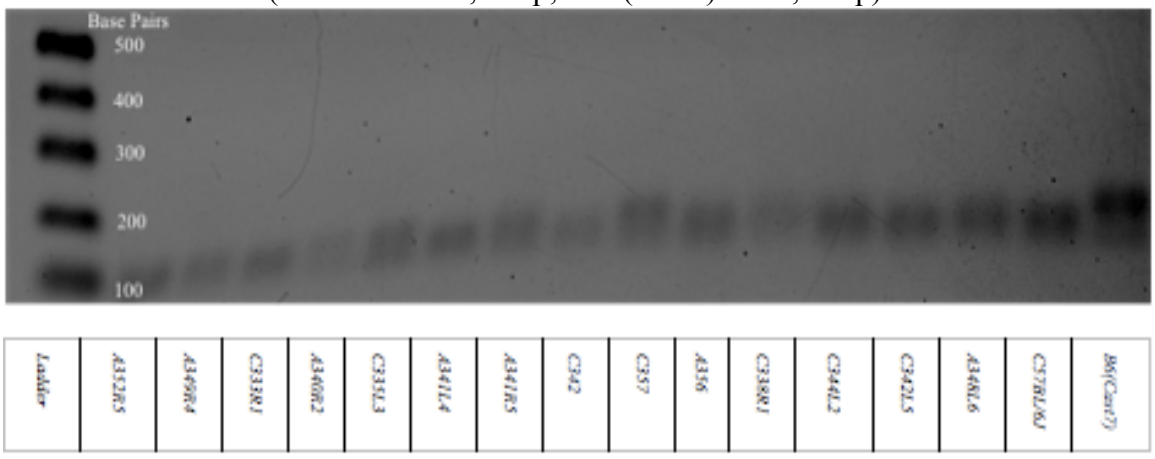
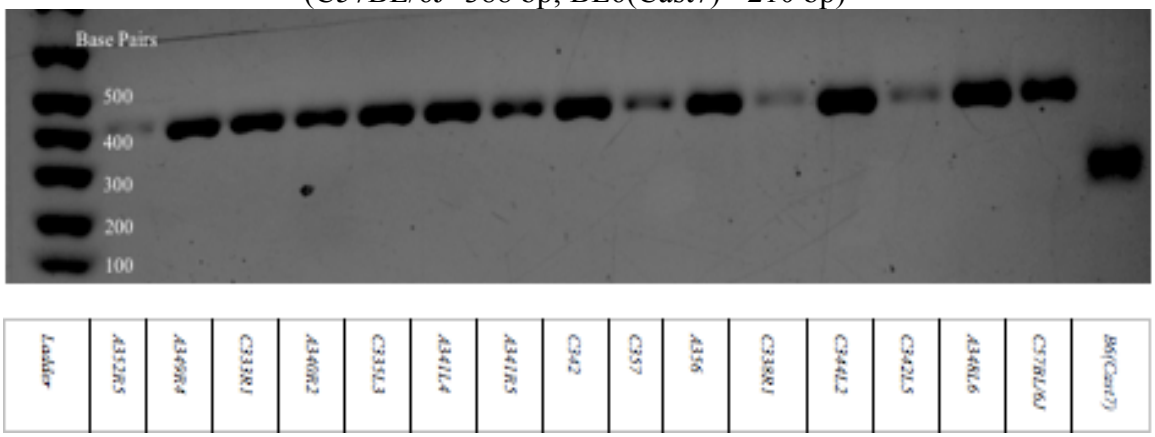


Figure 36. Continued

d

Igf2r Gene Expression
(C57BL/6J -388 bp; BL6(Cast7) - 210 bp)



e

Igf2 Gene Expression
(C57BL/6J -163 bp; BL6(Cast7) - 224 bp)

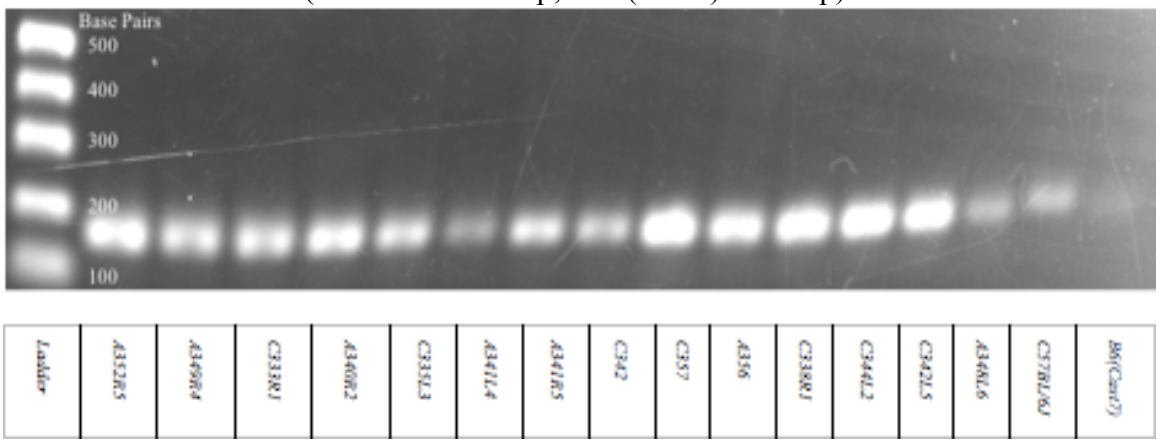
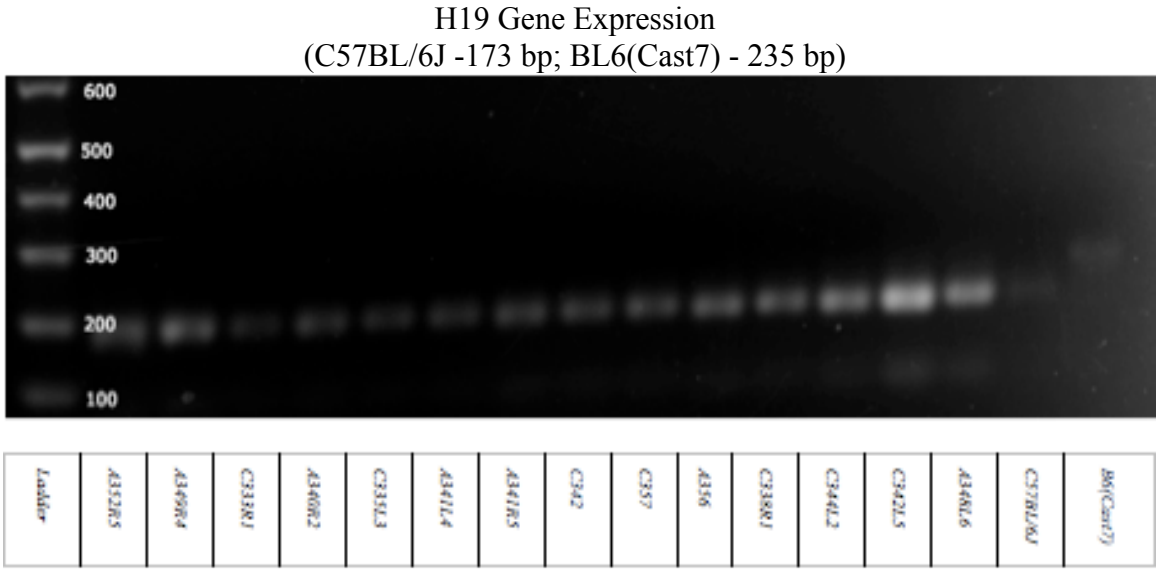


Figure 36. Continued

f



g

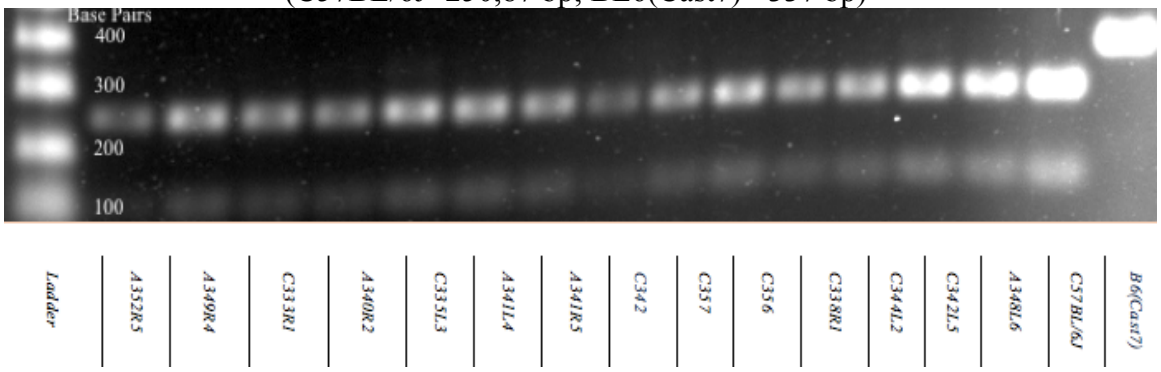


Figure 36. Continued

h

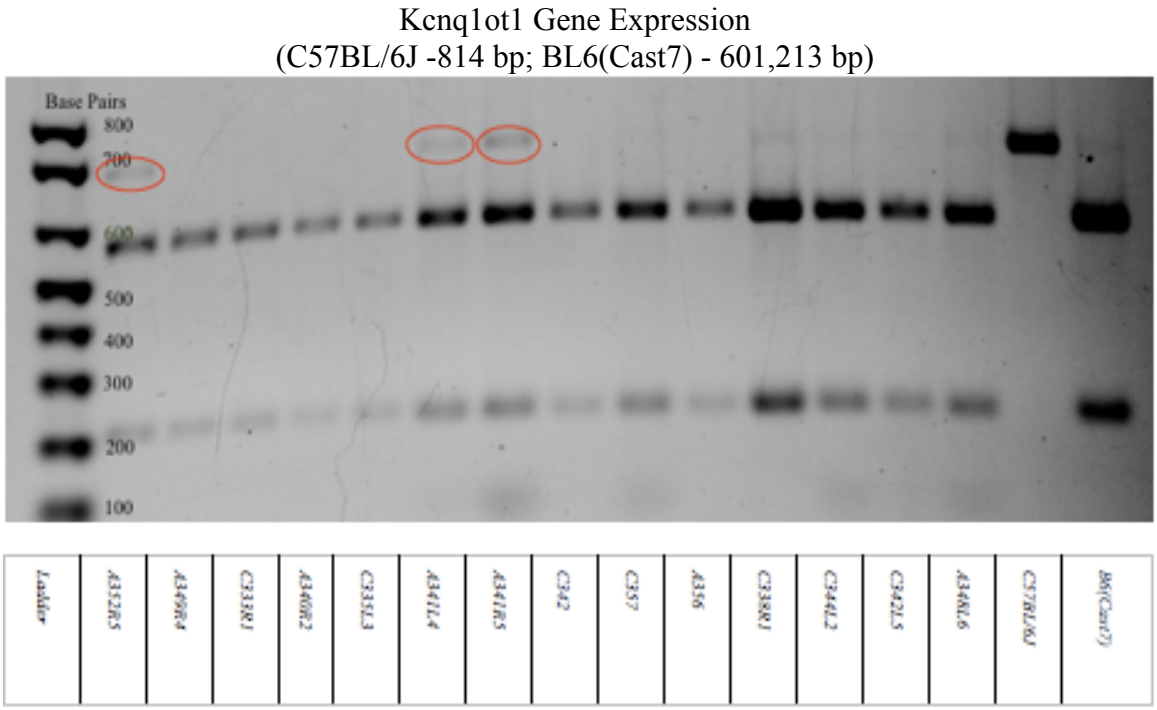


Figure 36. Continued

CHAPTER IV

DISCUSSION AND CONCLUSION

Epigenetics is the study of heritable changes produced from environmental factors. These factors include, but are not limited to, diet, exercise, stress and alcohol exposure. Epigenetics can alter the transcriptome through modifications leading to a difference in gene expression and influencing animal phenotype. By modifying the expression of chromatin, epigenetics is thought to alter which proteins are transcribed, ultimately dictating phenotypes. The difference between epigenetics and DNA is epigenetics is reversible and plastic, while DNA is non-reversible and static.

One of the mechanisms through which the environment influences transcription is through DNA methylation. DNA methylation is the most commonly studied epigenetic mechanism, as well as the first discovered to influence gene expression [23]. As previously stated, "DNA methylation is the addition of a methyl group on the fifth carbon of a cytosine nucleotide, to form 5-methylcytosine." These additions to the major grooves of DNA affect protein interaction significantly and influence how loose or tight the chromatin is regulating transcription and subsequently affect gene repression or activation [19,23]. Gene activation is associated with demethylated regions, whereas gene repression is associated with DNA methylation[26]. DNA methylation is commonly found on these short interspersed CpG islands through the genome[26].

DNA methyltransferases(DNMT) are responsible for regulating methylation. The family of DNMTs is comprised of DNMT1, DNMT2, DNMT3a, DNMT3b, and

DNMT3L. DNMT1 is known to maintain methylation within the genome, whereas DNMT3a and DNMT3b are known for *de novo* methylation [29,33], When these enzymes are misregulated, there can be catastrophic effects for the fetus, ranging from birth defects to death. While misregulation of DNA methylation is not always fatal, Rett Syndrome and cancer are associated with it [1,38–40].

DNA methylation is a well-studied factor in regulating genomic imprinting [55]. When genes are imprinted, one parental allele is relatively silent in comparison to the other allele. It is known that approximately 100 genes in mammals are imprinted [46]. It is easier to judge environmental effects by studying the disruptions of imprinted genes because only one allele is expressed [62]. Some environmental factors, such as alcohol, may disrupt imprinting.

Spermatogenesis is the process through which sperm are produced that includes three main phases: proliferation, meiosis, and differentiation. In mice, there are twelve stages, and four cycles culminating after 33 days[84]. Radiation, obesity, age and alcohol all can alter spermatogenesis, which can influence the development of offspring and fertility. Specifically, these factors can lead to decreased sperm morphology and motility, decreased semen volume and count, infertility, and misregulation of DNA methylation in sperm [86,87,89,90,92].

As previously stated, grandparents who survived starvation saw grandchildren more predisposed to obesity[94]. Studies have shown that the diet of the father does impact their offspring such as a low protein high fat diet and the association to increased

hepatic gene expression and a high fat diet and the relationship to female offspring having a elevated precursor to type 2 diabetes [97,99].

Another environmental factor such as alcohol, a known teratogen, can influence gene expression and misregulation of DNA methylation [103,104].

Fetal Alcohol Syndrome is very prevalent in society occurring with six to nine per 1000 live births [105] Maternal alcoholism has long been known to cause FAS, but much evidence exists to suggest that paternal alcoholism may also affect offspring. Given that paternal inherited genes are a major influence on the development of the placenta, it must be studied how the male consumption of alcohol affects offspring [59].

Through this study, we have shown that male mice chronically exposed to alcohol do influence fetal parameters. Four out of our five measurements had a significant difference in comparison to the control treatment. Gestational sac weight was not only significant when comparing weights between the two treatment groups ($p=0.0159$), but also the female offspring from the exposed male had a significant 7% reduction in weight in comparison to female offspring from the control group ($p=0.0095$). This indicates that there is a decrease in amniotic fluid in the ethanol treated offspring. In early gestation, the amnion plays a major role in the development of the fetus. Reducing the amnion could be associated with developmental disorders.

The second parameter that was significant was fetal weight. The fetal weight between the two treatment groups was significant with a 5% weight reduction in the experimental group ($p=0.0217$). While investigating the degree to which the fetuses were affected, we discovered there was a sex specific significant reduction in fetal weight. In

comparison to the male offspring from exposed male sires, the female offspring were significantly reduced in weight (p=0.0020). This indicates that the female offspring was more affected than the males. Supporting that is that there was no significance between the male fetal weight between the two treatment groups(p=0.4096). The female weight was significant when comparing it between the two treatment groups (p=0.0039).

We did find a significant difference the length of the fetuses between the two groups (p=0.0015), additionally there was sex specific significant difference within the experimental treatment group. There was a 3% significant reduction in female length in comparison to male length from the experimental treatment (p=0.0133). In addition, the placental weight between and within the treatment groups, was not significant. When using an alternate way to measure the placenta relative to the fetal weight, there was a significant difference in the treatment groups (p=0.0101). The experimental offspring had an 8% decrease in placenta efficiency. This data demonstrates a decrease in placenta efficiency and a decrease in fetal weight in the offspring from exposed sires. This may imply the placenta failed to transfer adequate nutrients to the fetus [129]. As before, there were was significant differences with the sexes between treatment groups with the females having a 14% significant decrease in placental efficiency, as well as a 12% significant difference in females compared to males sired by exposed males.

When observing the methylation patterns in the candidate genes in the sperm, none of the candidate regions examined a significant difference in percentage of methylation. The examination of gene expression through these genes as well showed no difference in expression between the treatment groups. However, when looking at other

imprinted genes in the same ICR, we did discover two genes that did have a differences in expression: Dio3 and Mirg ($p=0.0017$ and $p=0.0277$ respectively). Upon further examination of Dio3, the expression was sex specific. In the experimental treatment group, the expression of Dio3 in female offspring significantly increased ($p=0.0001$), and when comparing the females between the two treatment groups the females sired from exposed males were also significant ($p=0.0007$).

Although we did not find changes in gene expression in our candidate genes, we did observe two significant single nucleotide polymorphisms in H19 and Cdkn1c ($p<0.0001$ and $p=0.0436$ respectively). In H19 we did observe a significant decrease in transcripts from the paternal allele, whereas in Cdkn1c we observe an increase in the paternal allele that is normally silent. Loss of imprinting in Cdkn1c has been previously associated with a decrease in fetal growth [138]. Through the misregulation of the normal expression of the paternal allele in H19 and Cdkn1c, it does give support to the hypothesis that preconception male alcohol exposure can disrupt the expression of imprinted genes.

From the evidence shown, we conclude that paternal alcoholism is a significant contributor to characteristics of Fetal Alcohol Syndrome such as low birth weight. Popular culture predominantly focuses the anti-drinking message towards an expectant mother, but that message may have to be expanded to include fathers. Additionally, because of the impact alcohol has upon a developing fetus, the duration of sobriety couples must maintain may also extend before a conception. This particularly applies to

men as sperm is produced continuously in their adult life regenerating spermatozoan that can be effected months before fertilization from environmental factors such as alcohol.

REFERENCES

1. Allis CD, Jenuwein T, Reinberg D, Caparros M-L. Epigenetics. Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY:; 2007; Available: http://library.wur.nl/WebQuery/clc/1831507

2. Felsenfeld G. A brief history of epigenetics. Cold Spring Harb Perspect Biol. 2014;6. doi:10.1101/cshperspect.a018200

3. Muller HJ. Types of visible variations induced by X-rays in *Drosophila*. J Genet. 1930;22: 299–334.

4. McClintock B. Chromosome organization and genic expression. Cold Spring Harbor Symp Quant Biol. 1951; 13–47.

5. Ohno S, Kaplan WD, Kinosita R. Formation of the sex chromatin by a single X-chromosome in liver cells of *Rattus norvegicus*. Exp Cell Res. 1959;18: 415–418.

6. Lyon MF. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). Nature. 1961;190: 372–373.

7. Riggs AD. X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet. 1975;14: 9–25.

8. Doskočil J, Šorm F. Distribution of 5-methylcytosine in pyrimidine sequences of deoxyribonucleic acids. Biochimica et Biophysica Acta 1962;55: 953–959.

9. Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N. Requirement for Xist in X chromosome inactivation. Nature. 1996;379: 131–137.

10. Chow JC, Yen Z, Ziesche SM, Brown CJ. Silencing of the mammalian X chromosome. Annu Rev Genomics Hum Genet. 2005;6: 69–92.

11. Bantignies F, Cavalli G. Cellular memory and dynamic regulation of polycomb group proteins. Curr Opin Cell Biol. 2006;18: 275–283.

12. de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R, et al. Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell. 2004;7: 663–676.

13. Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, et al. Role of histone H3 lysine 27 methylation in X inactivation. Science. 2003;300: 131–135.

14. Waddington CH. The epigenotype. 1942. Int J Epidemiol. 2012;41: 10–13.

15. Klar AJ. Propagating epigenetic states through meiosis: where Mendel's gene is more than a DNA moiety. Trends Genet. 1998;14: 299–301.

16. Watson JD. Celebrating the genetic jubilee: a conversation with James D. Watson. interviewed by John Rennie. Sci Am. 2003;288: 66–69.

17. Ptashne M. On the use of the word "epigenetic." Curr Biol. 2007;17: R233–6.

18. Kornberg RD, Lorch Y. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell. 1999;98: 285–294.

19. Smolle M, Workman JL. Transcription-associated histone modifications and cryptic transcription. Biochim Biophys Acta. 2013;1829: 84–97.

20. Feinberg AP. Epigenetics at the epicenter of modern medicine. JAMA. 2008;299: 1345–1350.

21. Kappil M, Lambertini L, Chen J. Environmental influences on genomic imprinting. Curr Environ Health Rep. 2015;2: 155–162.

22. Burd L, Roberts D, Olson M, Odendaal H. Ethanol and the placenta: A review. J Matern Fetal Neonatal Med. 2007;20: 361–375.

23. Razin A, Cedar H. DNA methylation and gene expression. Microbiol Rev. 1991;55: 451–458.

24. Zakhari S. Alcohol metabolism and epigenetics changes. Alcohol Res. 2013;35: 6–16.

25. Larsen F, Gundersen G, Lopez R, Prydz H. CpG islands as gene markers in the human genome. Genomics. 1992;13: 1095–1107.

26. Naveh-Many T, Cedar H. Active gene sequences are undermethylated. Proc Natl Acad Sci U S A. 1981;78: 4246–4250.

27. Hausheer FH, Rao SN, Gamcsik MP, Kollman PA, Colvin OM, Saxe JD, et al. Computational analysis of structural and energetic consequences of DNA methylation. Carcinogenesis. 1989;10: 1131–1137.

28. Boyes J, Bird A. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell. 1991;64: 1123–1134.

29. Bestor T, Laudano A, Mattaliano R, Ingram V. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. J Mol Biol. 1988;203: 971–983.

30. Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell. 1992;69: 915–926.

31. Schaefer M, Pollex T, Hanna K, Tuorto F, Meusburger M, Helm M, et al. RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. Genes Dev. 2010;24: 1590–1595.

32. Tuorto F, Herbst F, Alerasool N, Bender S, Popp O, Federico G, et al. The tRNA methyltransferase Dnmt2 is required for accurate polypeptide synthesis during haematopoiesis. EMBO J. 2015;34: 2350–2362.

33. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999;99: 247–257.

34. Ehrlich M. The ICF syndrome, a DNA methyltransferase 3B deficiency and immunodeficiency disease. Clin Immunol. 2003;109: 17–28.

35. Hata K, Okano M, Lei H, Li E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development. 2002;129: 1983–1993.

36. Bourc'his D, Bestor TH. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature. 2004;431: 96–99.

37. Chédin F, Lieber MR, Hsieh C-L. The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. Proceedings of the National Academy of Sciences. 2002;99: 16916–16921.

38. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet. 2002;3: 415–428.

39. Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet. 1999;21: 163–167.

40. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. 2003;349: 2042–2054.

41. Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M. Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res. 1988;48: 1159–1161.

42. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature. 1983;301: 89–92.

43. Cooper DN, Youssoufian H. The CpG dinucleotide and human genetic disease. Hum Genet. 1988;78: 151–155.

44. DNA Damage and Repair. In: Sigma-Aldrich [Internet]. [Accessed 23 Jun 2016]. Available: http://www.sigmaaldrich.com/technical-documents/articles/biofiles/dna-damage-and-repair.html

45. McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell. 1984;37: 179–183.

46. Bartolomei MS, Ferguson-Smith AC. Mammalian genomic imprinting. Cold Spring Harb Perspect Biol. 2011;3. doi:10.1101/cshperspect.a002592

47. Barlow DP, Stöger R, Herrmann BG, Saito K, Schweifer N. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. Nature. 1991;349: 84–87.

48. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. Cell. 1991;64: 849–859.

49. Bartolomei MS, Zemel S, Tilghman SM. Parental imprinting of the mouse H19 gene. Nature. 1991;351: 153–155.

50. Lyle R, Watanabe D, te Vruchte D, Lerchner W, Smrzka OW, Wutz A, et al. The imprinted antisense RNA at the Igf2r locus overlaps but does not imprint Mas1. Nat Genet. 2000;25: 19–21.

51. Schmidt JV, Matteson PG, Jones BK, Guan XJ, Tilghman SM. The Dlk1 and Gtl2 genes are linked and reciprocally imprinted. Genes Dev. 2000;14: 1997–2002.

52. Lee MP, DeBaun MR, Mitsuya K, Galonek HL, Brandenburg S, Oshimura M, et al. Loss of imprinting of a paternally expressed transcript, with antisense orientation to KVLQT1, occurs frequently in Beckwith-Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. Proc Natl Acad Sci U S A. 1999;96: 5203–5208.

53. Zhao J, Ohsumi TK, Kung JT, Ogawa Y, Grau DJ, Sarma K, et al. Genome-wide identification of polycomb-associated RNAs by RIP-seq. Mol Cell. 2010;40: 939–953.

54. Kung JTY, Colognori D, Lee JT. Long noncoding RNAs: past, present, and future. Genetics. 2013;193: 651–669.

55. Fowden AL, Coan PM, Angiolini E, Burton GJ, Constancia M. Imprinted genes and the epigenetic regulation of placental phenotype. Prog Biophys Mol Biol. 2011;106: 281–288.

56. Bartolomei MS. Genomic imprinting: employing and avoiding epigenetic processes. Genes Dev. 2009;23: 2124–2133.

57. Saitou M, Kagiwada S, Kurimoto K. Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development. 2012;139: 15–31.

58. Santos F, Hendrich B, Reik W, Dean W. Dynamic Reprogramming of DNA methylation in the early mouse embryo. Dev Biol. 2002;241: 172–182.

59. Monk D. Genomic imprinting in the human placenta. Am J Obstet Gynecol. 2015;213: S152–62.

60. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. Nat Rev Genet. 2007;8: 253–262.

61. Hitchins MP. Constitutional epimutation as a mechanism for cancer causality and heritability? Nat Rev Cancer. 2015;15: 625–634.

62. Wang S, Yu Z, Miller RL, Tang D, Perera FP. Methods for detecting interactions between imprinted genes and environmental exposures using birth cohort designs with mother-offspring pairs. Hum Hered. 2011;71: 196–208.

63. Doherty AS, Mann MR, Tremblay KD, Bartolomei MS, Schultz RM. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. Biol Reprod. 2000;62: 1526–1535.

64. Mann MRW, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM, et al. Selective loss of imprinting in the placenta following preimplantation development in culture. Development. 2004;131: 3727–3735.

65. Haycock PC, Ramsay M. Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the H19 imprinting control region. Biol Reprod. 2009;81: 618–627.

66. Susiarjo M, Sasson I, Mesaros C, Bartolomei MS. Bisphenol a exposure disrupts genomic imprinting in the mouse. PLoS Genet. 2013;9: e1003401.

67. Adams J. Imprinting and genetic disease: Angelman, Prader-Willi and Beckwith-Weidemann syndromes. Nat Educ. 2008;1: 129.

68. Down JL. On some of the mental affections of childhood and youth. London : Mac Keith Press; 1887.

69. Prader, A. Ein syndrome von Adipositas, Kleinwuchs, Kryptorchismus und Oligophreniee nach myatonieartigerm Zustand im Neugeborenenalter. Schweiz Med Wochenschr. 1956;86: 1260–1261.

70. Prader-Willi Syndrome. In: Genetics Home Reference [Internet]. U.S. National Library of Medicine; 7 Mar 2016. Available: https://ghr.nlm.nih.gov/condition/prader-willi-syndrome

71. Wiedemann H-R. Living history-autobiography: Hans-Rudolf Wiedemann in a half century of german pediatric genetics. Am J Med Genet. 1992;43: 740–746.

72. Beckwith-Wiedemann syndrome. In: Genetics Home Reference [Internet]. U.S. National Library of Medicine; 7 Mar 2016. Available: https://ghr.nlm.nih.gov/condition/beckwith-wiedemann-syndrome

73. Henry I, Bonaiti-Pellié C, Chehensse V, Beldjord C, Schwartz C, Utermann G, et al. Uniparental paternal disomy in a genetic cancer-predisposing syndrome. Nature. 1991;351: 665–667.

74. Tanner JM, Ham TJ. Low birthweight dwarfism with asymmetry (Silver's syndrome): treatment with human growth hormone. Arch Dis Child. 1969;44: 231–243.

75. Eggermann T, Begemann M, Binder G, Spengler S. Silver-Russell syndrome: genetic basis and molecular genetic testing. Orphanet J Rare Dis. 2010;5: 1–8.

76. Russell-Silver syndrome. In: Genetics Home Reference [Internet]. U.S. National Library of Medicine; 7 Mar 2016. Available: https://ghr.nlm.nih.gov/condition/russell-silver-syndrome

77. Angelman Syndrome - Pictures, Facts, Life Expectancy, Treatment [Internet]. [Accessed 23 Jun 2016]. Available: http://healthh.com/angelman-syndrome/

78. Beckwith-Wiedemann syndrome: MedlinePlus Medical Encyclopedia Image [Internet]. [Accessed 23 Jun 2016]. Available: https://www.nlm.nih.gov/medlineplus/ency/imagepages/17076.htm

79. Thulasi. Prader Willi Syndrome - Symptoms, Causes, Complications, Pictures | Diseases Lab [Internet]. [Accessed 24 Jun 2016]. Available: http://diseaseslab.com/prader-willi-syndrome-pictures-symptoms-causes-treatment/

80. Russell-Silver Syndrome. In: Manbir Online ~ Diseases & Conditions [Internet]. 23 Apr 2011 [Accessed 24 Jun 2016]. Available: https://molconditions.wordpress.com/2011/04/23/russell-silver-syndrome/

81. Gilbert SF. Structure of the Gametes. Sinauer Associates; 2000.

82. Biology: Textbook for Class XII. National Council of Education Research and Training; 2006.

83. Sobarzo C, Bustos Obregón E. Acute effect of parathion on the seminiferous epithelium of immature mice. Rev chil anat. bases.bireme.br; 2000;18: 61–68.

84. Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci. 1952;55: 548–573.

85. Russell LD, Ettlin RA, Hikim APS, Clegg ED. Histological and histopathological evaluation of the testis. Int J Androl. 1993;16: 83–83.

86. Howell SJ, Shalet SM. Article spermatogenesis after cancer treatment: damage and recovery. J Natl Cancer Inst Monogr. Citeseer; 2005;34: 12–17.

87. Auger J, Kunstmann JM, Czyglik F, Jouannet P. Decline in semen quality among fertile men in Paris during the past 20 years. N Engl J Med. 1995;332: 281–285.

88. Schwartz D, Mayaux MJ, Spira A, Moscato ML, Jouannet P, Czyglik F, et al. Semen characteristics as a function of age in 833 fertile men. Fertil Steril. 1983;39: 530–535.

89. Lambrot R, Xu C, Saint-Phar S, Chountalos G, Cohen T, Paquet M, et al. Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. Nat Commun. 2013;4: 2889.

90. Donkin I, Versteyhe S, Ingerslev LR, Qian K, Mechta M, Nordkap L, et al. Obesity and bariatric surgery drive epigenetic variation of spermatozoa in humans. Cell Metab. 2015; doi:10.1016/j.cmet.2015.11.004

91. Muthusami KR, Chinnaswamy P. Effect of chronic alcoholism on male fertility hormones and semen quality. Fertil Steril. 2005;84: 919–924.

92. Liang F, Diao L, Jiang N, Zhang J, Wang H-J, Zhou W-H, et al. Chronic exposure to ethanol in male mice may be associated with hearing loss in offspring. Asian J Androl. 2015;17: 985–990.

93. Rajender S, Avery K, Agarwal A. Epigenetics, spermatogenesis and male infertility. Mutat Res. 2011;727: 62–71.

94. Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjöström M, et al. Sex-specific, male-line transgenerational responses in humans. Eur J Hum Genet. 2006;14: 159–166.

95. Barker DJ. Fetal origins of coronary heart disease. BMJ. 1995;311: 171–174.

96. Barker DJP, Osmond C. Infant mortality, childhood nutrition, and ischemic heart disease in England and Wales. Lancet. 1986;327: 1077–1081.

97. Carone BR, Fauquier L, Habib N, Shea JM, Hart CE, Li R, et al. Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. Cell. 2010;143: 1084–1096.

98. Ng S-F, Lin RCY, Laybutt DR, Barres R, Owens JA, Morris MJ. Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. Nature. 2010;467: 963–966.

99. Murashov AK, Pak ES, Koury M, Ajmera A, Jeyakumar M, Parker M, et al. Paternal long-term exercise programs offspring for low energy expenditure and increased risk for obesity in mice. FASEB J. 2015; doi:10.1096/fj.15-274274

100. Rodgers AB, Morgan CP, Bronson SL, Revello S, Bale TL. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. J Neurosci. Soc Neuroscience; 2013;33: 9003–9012.

101. White SL, Vassoler FM, Schmidt HD, Pierce RC, Wimmer ME. Enhanced anxiety in the male offspring of sires that self-administered cocaine. Addict Biol. 2015; doi:10.1111/adb.12258

102. Lee K-M, Ward MH, Han S, Ahn HS, Kang HJ, Choi HS, et al. Paternal smoking, genetic polymorphisms in CYP1A1 and childhood leukemia risk. Leuk Res. Elsevier; 2009;33: 250–258.

103. Kojima T, Asano S, Takahashi N. Teratogenic factors affect transcription factor expression. Biosci Biotechnol Biochem. 2013;77: 1035–1041.

104. Garro AJ, Espina N, McBeth D, Wang SL, Wu-Wang CY. Effects of alcohol consumption on DNA methylation reactions and gene expression: implications for increased cancer risk. Eur J Cancer Prev. 1992;1 Suppl 3: 19–23.

105. May PA, Baete A, Russo J, Elliott AJ, Blankenship J, Kalberg WO, et al. Prevalence and characteristics of fetal alcohol spectrum disorders. Pediatrics. 2014;134: 855–866.

106. Alcohol Research & Health, Volume 34, Number 1 [Internet]. [Accessed 24 Jun 2016]. Available: http://pubs.niaaa.nih.gov/publications/arh341/4-14.htm

107. Esser MB, Hedden SL, Kanny D, Brewer RD, Gfroerer JC, Naimi TS. Prevalence of alcohol dependence among US adult drinkers, 2009-2011. Prev Chronic Dis. 2014;11: E206.

108. Naimi TS, Brewer RD, Mokdad A, Denny C, Serdula MK, Marks JS. Binge drinking among US adults. JAMA. 2003;289: 70–75.

109. NIAAA Newsletter, Winter 2004, Issue 3. Available: http://pubs.niaaa.nih.gov/publications/Newsletter/winter2004/Newsletter_Number3.pdf

110. Passaro KT, Little RE, Savitz DA, Noss J. Effect of paternal alcohol consumption before conception on infant birth weight. Teratology. 1998;57: 294–301.

111. Hegedus AM, Alterman AI, Tarter RE. Learning achievement in sons of alcoholics. Alcohol Clin Exp Res. 1984;8: 330–333.

112. Tarter RE, Hegedus AM, Goldstein G, Shelly C, Alterman AI. Adolescent sons of alcoholics: neuropsychological and personality characteristics. Alcohol Clin Exp Res. 1984;8: 216–222.

113. Meek LR, Myren K, Sturm J, Burau D. Acute paternal alcohol use affects offspring development and adult behavior. Physiol Behav. 2007;91: 154–160.

114. Abel E. Paternal contribution to fetal alcohol syndrome. Addict Biol. 2004;9: 127–33; discussion 135–6.

115. Ledig M, Misslin R, Vogel E, Holownia A, Copin JC, Tholey G. Paternal alcohol exposure: developmental and behavioral effects on the offspring of rats. Neuropharmacology. 1998;37: 57–66.

116. Curley JP, Mashoodh R, Champagne FA. Epigenetics and the origins of paternal effects. Horm Behav. 2011;59: 306–314.

117. Lee JT, Bartolomei MS. X-Inactivation, imprinting, and long noncoding RNAs in health and disease. Cell. 2013;152: 1308–1323.

118. Piedrahita JA. The role of imprinted genes in fetal growth abnormalities. Birth Defects Res A Clin Mol Teratol. 2011;91: 682–692.

119. Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC. Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol Behav. 2005;84: 53–63.

120. Brady ML, Allan AM, Caldwell KK. A limited access mouse model of prenatal alcohol exposure that produces long-lasting deficits in hippocampal-dependent learning and memory. Alcohol Clin Exp Res. 2012;36: 457–466.

121. Mann MRW, Chung YG, Nolen LD, Verona RI, Latham KE, Bartolomei MS. Disruption of imprinted gene methylation and expression in cloned preimplantation stage mouse embryos. Biol Reprod. 2003;69: 902–914.

122. Parreira GG, Ogawa T, Avarbock MR, França LR, Brinster RL, Russell LD. Development of germ cell transplants in mice. Biol Reprod. 1998;59: 1360–1370.

123. Blood Alcohol Concentration (BAC). Available: http://www.cdc.gov/motorvehiclesafety/pdf/BAC-a.pdf

124. França LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD. Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol Reprod. 1998;59: 1371–1377.

125. Golding MC, Magri LS, Zhang L, Lalone SA, Higgins MJ, Mann MRW. Depletion of Kcnq1ot1 non-coding RNA does not affect imprinting maintenance in stem cells. Development. 2011;138: 3667–3678.

126. Knezovich JG, Ramsay M. The effect of preconception paternal alcohol exposure on epigenetic remodeling of the h19 and rasgrf1 imprinting control regions in mouse offspring. Front Genet. 2012;3: 10.

127. Gundogan F, Elwood G, Longato L, Tong M, Feijoo A, Carlson RI, et al. Impaired placentation in fetal alcohol syndrome. Placenta. 2008;29: 148–157.

128. Lager S, Samulesson A-M, Taylor PD, Poston L, Powell TL, Jansson T. Diet-induced obesity in mice reduces placental efficiency and inhibits placental mTOR signaling. Physiol Rep. 2014;2: e00242.

129. Hayward CE, Lean S, Sibley CP, Jones RL, Wareing M, Greenwood SL, et al. Placental adaptation: what can we learn from birthweight:placental weight ratio? Front Physiol. 2016;7: 28.

130. Chen S, Sun F-Z, Huang X, Wang X, Tang N, Zhu B, et al. Assisted reproduction causes placental maldevelopment and dysfunction linked to reduced fetal weight in mice. Sci Rep. 2015;5: 10596.

131. Huang L, Liu J, Feng L, Chen Y, Zhang J, Wang W. Maternal prepregnancy obesity is associated with higher risk of placental pathological lesions. Placenta. 2014;35: 563–569.

132. Zalenskaya IA, Bradbury EM, Zalensky AO. Chromatin structure of telomere domain in human sperm. Biochem Biophys Res Commun. 2000;279: 213–218.

133. Krueger F, Kreck B, Franke A, Andrews SR. DNA methylome analysis using short bisulfite sequencing data. Nat Methods. 2012;9: 145–151.

134. Hisano M, Erkek S, Dessus-Babus S, Ramos L, Stadler MB, Peters AHFM. Genome-wide chromatin analysis in mature mouse and human spermatozoa. Nat Protoc. 2013;8: 2449–2470.

135. Battaglia FC, Regnault TR. Placental transport and metabolism of amino acids. Placenta. 2001;22: 145–161.

136. Díaz P, Powell TL, Jansson T. The role of placental nutrient sensing in maternal-fetal resource allocation. Biol Reprod. 2014;91: 82.

137. Sittig LJ, Shukla PK, Herzing LBK, Redei EE. Strain-specific vulnerability to alcohol exposure in utero via hippocampal parent-of-origin expression of deiodinase-III. FASEB J. 2011;25: 2313–2324.

138. Fitzpatrick GV, Soloway PD, Higgins MJ. Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. Nat Genet. 2002;32: 426–431.